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Acquired immunity against intracellular bacteria is T cell dependent. T cells play a major role in protection against intracellular bacteria, but bacterial antigens recognized by T cells have been studied less extensively than bacterial antigens recognized by B cells. Using T lymphocytes from *Brucella abortus* immunized animals, we have screened a bacterial genomic library for genes encoding antigens recognized by T cells. Lymphocytes that proliferated to *B. abortus* proteins were characterized for phenotype and cytokine activity. Lymphocytes recognizing bacterial antigens possessed type 1 cytokine profiles, i.e., IFN-γand IL-2 but not IL-4 production. Lymphocytes necesssary for *In vivo* protection were examined using gene knockout mice. MHC class I and class II gene knockout animals infected with *B. abortus* demonstrated that protection to *B. abortus* is especially dependent on $CD8^+$ T cells. Knowing the cells required for protection, vaccines can be designed to elicit the protective subset of lymphocytes. Several recombinant *B. abortus* proteins were tested using different immunization strategies.

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Gary Peltier — May 20, 1996

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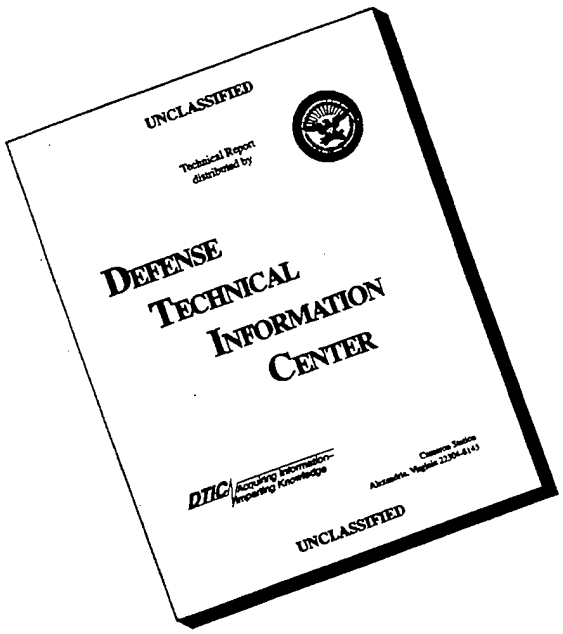

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INTRODUCTION

1. The Problem

Brucella melitensis is a gram-negative facultative intracellular bacterium that induces chronic infectious disease by direct contact or by consumption of animal products. The intracytoplasmic localization likely accounts for the great chronicity of the disease; many patients remain ill for months to years. Although the genus *Brucella* consists of six species that exhibit preferential host adaptation (Thoen and Enright, 1986), greater than 98% homology of various *Brucella* species occurs at the DNA level (Verger, et al., 1987). Therefore, proteins encoded by the DNA must be very similar, if not identical. In fact *Brucella melitensis*, *B. suis* and *B. abortus* can all infect humans with similar serious disease consequences. Natural infections occur when the organisms penetrate the mucosa of the nasal, oral or pharyngeal cavities. Following penetration, the bacteria are transported either free or within phagocytic cells to regional lymph nodes where hyperplasia and inflammation occur. Secondary localization in other lymph nodes, spleen, liver, and bone where granulomas form. Viable bacilli may persist for several months in the granulomas, giving rise to acute or chronic symptoms. For this reason the incubation period in brucellosis may be very long.

Two species, *B. abortus* and *B. melitensis* have particular relevance to humans. *B. abortus* primarily affects cattle causing abortion or the birth of severely debilitated calves and contaminated dairy products serve as a source for human infection. *B. melitensis* is frequently the cause of human brucellosis in countries other than the United States and is found particularly in the Mediterranean region.

Brucella infects humans and replicates in mononuclear phagocytes. In humans, the pathological manifestations of brucellosis include arthritis, endocarditis, and meningitis. Chronic infection is a hallmark. Immunity to *B. abortus* is dependent on cell-mediated immune responses. T lymphocyte engagement in response to *Brucella* proteins appears to be a decisive cell-mediated mechanism to provide resistance to *B. abortus* infection. Therefore, identifying bacterial proteins that induce a T lymphocyte-mediated response is critical for understanding immunity to brucellosis.

Of particular concern to the military, *B. melitensis* has potential use as a biologic warfare agent against U.S. troops. Presently, a human vaccine does not exist. Thus, there is an immediate need to develop a functional, practical vaccine that affords protection in the field to military personnel.

2. Background of Previous Work

Since the early experiments of Mackaness with *Listeria* (Mackaness, 1964) and others with *B. abortus* (Halliburton and Hinsdill, 1972), cellular immunity is assumed to have an important role in brucellosis. Macrophages serve as key cells in maintaining the facultative intracellular bacterium and in presenting *Brucella* antigens to T lymphocytes for the initiation of an immune response.

Several lines of evidence suggest that cellular immunity limits the pathogenesis of brucellosis. The preferential migration of *Brucella*-specific bovine lymphocytes from a primary challenged lymph node to a secondarily challenged site confirms the ability of T lymphocytes specific for *Brucella* epitopes to participate in the in vivo immune response (Liu and Splitter, 1986). Macrophages serve as important resident cells for *B. abortus*, and these cells phagocytize and kill most ingested species of bacteria within a few hours (Bermudez and Young, 1988; Spitalny and North, 1981). Resistance depends on successful interaction between T lymphocytes specific for

particular bacterial antigens and macrophages (Fiorentino *et al.*, 1991). *Brucella* proteins that induce a T lymphocyte mediated response are most likely responsible for the observed protection.

Vaccination *B. abortus* strain 19 usually results in a transient rise in antibodies specific for the bacterial lipopolysaccharide (LPS). Passive transfer of anti-LPS antibody alone is not protective, although some protection is afforded experimentally in mice (Araya *et al.*, 1989). Greater protection to *B. abortus* has been shown in mice by adoptive transfer of $CD4^+$ and $CD8^+$ lymphocytes (Araya *et al.*, 1989; Pavlov *et al.*, 1982), but similar adoptive lymphocyte transfer experiments have not been possible in humans. Also, recent information (Mielke, 1991) as well as our own published work (Oliveira and Splitter, 1995) suggests in the mouse that $CD8^+$ lymphocytes are more important than $CD4^+$ lymphocytes in the resolution of brucellosis infection.

Therefore, important aims in characterizing host resolution of brucellosis are: 1. identifying intracellular bacterial antigens recognized by T lymphocytes, 2. determining T lymphocyte subsets that respond to bacterial antigens, 3. determining the immunogenicity of recombinant bacterial proteins, and 4. a strategy for identifying bacterial virulence factors involved with intracellular survival of brucella.

3. Purpose of the Present Work

Our overall aim was to identify the immunodominant *Brucella* proteins important for T lymphocyte responses. The specific objectives were to:

a. Identify and define by DNA sequence analysis *Brucella melitensis* antigens recognized by T lymphocytes.

b. Express the recombinant bacterial proteins and verify that T lymphocytes from mice recognize the recombinant protein.

BODY

1. Intracellular bacterial antigens identified by T lymphocytes

We have identified *Brucella* proteins that stimulate lymphocyte proliferation using two approaches (Brooks-Worrell and Splitter,1992a; Brooks-Worrell and Splitter, 1992b; Brooks-Alder and Splitter, 1988; Zhu, *et al.*, 1993; Oliveira, *et al.*, 1994). First, individual *B. abortus* proteins that stimulated lymphocyte proliferation were characterized by one- and two-dimensional cellular immunoblotting, and thirty-eight *Brucella* proteins that induced lymphocyte proliferation were resolved (Brooks-Worrell and Splitter,1992). In addition, lymphocytes from strain 19 vaccinated animals proliferated in response to proteins isolated from other *Brucella* species (Brooks-Worrell and Splitter, 1992b). These results suggest that a genus-wide subunit vaccine may be feasible.

Two immunogenic proteins, 12 and 31 kDa were subjected to partial N-terminal amino acid analysis. An oligonucleotide probe was constructed that successfully identified the *L7/L12* gene (GenBank #L23505) and associated *L10* gene (GenBank #L19101) that comprise the rplJL operon (Oliveira, *et al.*, 1994b). These genes encode the L10 and L7/L12 proteins, essential for bacterial ribosomal function and protein synthesis. We then confirmed that the L7/L12 protein represents the immunodominant 12 kDa protein previously identified on immunoblotting (Oliveira and Splitter, 1994). The L7/L12 protein from *Mycobacterium bovis* (Tantimavanich, 1993) and *Brucella*

melitensis (Brachrach, 1994) provoked a strong delayed type hypersensitivity response in primed guinea pigs. Recently, others (Skeiky, 1995) have characterized a recombinant *Leishmania braziliensise* elF4A ribosomal protein as a potent antigen stimulating a strong Th1 lymphocyte response as well as IL-12 production in peripheral blood mononuclear cells from *Leishmania*-infected patients. Historically, ribosomal vaccines have been prepared from over 28 different bacteria, conferring a high degree of protection yet the reasons for protection have not been explained (Gregory, 1986). Why ribosomal proteins are a major stimulant of T lymphocyte response is unclear, but the levels of these proteins vary directly with the growth rate of the bacterium (Abshire and Neidhardt, 1993). In fact, *Brucella* ribosomal preparations have shown protection equal to the current strain 19 vaccination (Corbel, 1976), and now we have shown that L7/L12 is protective in mice.

Our second approach to identify immunogenic proteins that stimulate bovine lymphocytes utilizes *B. abortus* S2308 proteins expressed from a pBluescript II SK$^-$ genomic library (Zhu, *et al.*, 1993). Individual bacterial colonies were isolated and gene expression induced using isopropylthiogalactoside overnight and then bacterial culture supernatant was added to bovine peripheral blood mononuclear cells from animals primed to strain 19. This novel approach identified the *B. abortus uvrA* gene (GenBank #L10843) as stimulatory to T lymphocytes in all three animals tested, suggesting that it may represent a second immunodominant antigen. Using the same methodology, we have recently identified another two proteins, transporters containing ATP binding domains, that stimulate T cell proliferation from several animals. The genes encoding these proteins has been cloned and one of them fully sequenced, and appear to be members of the hemolysin β family of proteins found in *Escherichia coli*. These genes likely encode transporter proteins that conveys proteins outside or inside the bacterium. In *E. coli* a similar transport protein carries the hemolysin β protein, which is a serious human toxin. We have not yet found a hemolysin toxin in *Brucella*.

Using lymphocytes that respond to *Brucella* proteins expressed from single recombinant *E. coli* clones provides a novel strategy for identifying an array of candidates for protective molecular vaccines. The identification of *B. abortus* proteins immunodominant for T lymphocytes is an important first step in selecting *Brucella* proteins that may compose a subunit vaccine. Also, understanding the immune function of bacterial genes and their encoded proteins provides the opportunity to discern in greater detail the host-pathogen relationship.

2. T lymphocyte subsets that respond to bacterial antigens

To determine the T lymphocyte subsets that respond to the antigens of this intracellular bacterium, the L7/L12 protein was used as an example. The *B. abortus L7/L12* ribosomal gene was amplified by PCR and subcloned into the prokaryotic expression vector pMAL-c2. *E. coli* DH5α was transformed with the pMAL-L7/L12 construct and gene expression was induced by isopropylthiogalactoside. The resulting fusion protein was purified by affinity chromatography and confirmed by Western blot analysis using anti-maltose binding protein (MBP) antibody. Purified recombinant (r) L7/L12 protein induced proliferation of *B. abortus* primed T lymphocytes. Phenotypic analysis of the proliferating cell population following 7 days of culture demonstrated a two-fold increase in the percentage of CD4$^+$ T lymphocytes. No alteration in the proportion of CD8$^+$, IgM$^+$or γδ$^+$ T lymphocytes was observed (Oliveira, and Splitter, 1994).

Because mice have been used as a predictive model of cellular immune response to *Brucella* infection, we have examined the pattern of T helper (Th) cell response from infected BALB/c mice after *in vitro* stimulation with rL7/L12 ribosomal protein or γ-irradiated *B. abortus*. In addition to antigen specific proliferation, $CD4^+$ T cells were tested for IL-2, IL-4 and IFN-γ mRNA expression and secretion. Detection of cytokine transcripts and secreted cytokines was performed using reverse transcriptase (RT)-PCR and specific ELISA assays. Primed murine $CD4^+$ T cells, similar to bovine cells proliferated either to recombinant L7/L12 or whole *B. abortus*. The functional cytokine profile of the proliferating cells was typical of a type1 cytokine phenotype, as we detected transcripts for IL-2 and IFN-γ, but not IL-4. Among the cytokines analyzed, only IFN-γ was detected by ELISA assay in the $CD4^+$ T cell culture supernatants when bacteria or recombinant protein were used. Thus, rL7/L12 ribosomal protein and γ-irradiated *B. abortus* preferentially stimulate IFN-γ producing Th1 cells after *in vitro* stimulation (Oliveira, *et al.*, 1994a). These results provide for the first time an explanation why ribosomal vaccines may protect against intracellular infections. Moreover, this provides an experimental model for identifying pathogen derived polypeptides which stimulate the desired cytokine profile and Th cell subset response crucial for the design of protective vaccines.

As an inital step in the identification of bacterial proteins that mediate cellular immmunity, we have subcloned the *B. abortus ssb, uvrA, GroES*, and *GroEL* genes into the prokaryotic expression vector pMAL-c2 using PCR. *Escherichia coli* DH5α was transformed with the pMAL-ssb, pMAL-uvrA, pMAL-GroES, and pMAL-GroEL constructs separately, and gene expression was induced by isopropyl-β-D-thiogalactopyranoside. The resulting fusion proteins were purified by affinity chromatography and confirmed by Western blot analysis using an anti-maltose-binding protein antibody. Furthermore, we have examined the pattern of T helper cell response from vaccinated BALB/c mice after in vitro stimulation with the recombinant fusion proteins. In addition, to T-cell proliferative responses, $CD4^+$ T cells were tested for IL-2, IL-4, and IFN-γ secretion. Primed $CD4^+$ T cells proliferated to the rUvra, rGroES, and rGroEL, but not to rSsb. The cytokine profile of the proliferating cells was characteristic of a Th1 type, as we detected IL-2 and IFN-γ but not IL-4 in the T-cell culture supernatants. The recombinant *B. abortus* proteins were also screened in vivo for their ability to elicit DTH reaction in *Brucella*-sensitized guinea pigs. Moreover, the results of this study suggest that *B. abortus* rUvrA, rGroES and rGroEL might be important sources of potentially protective molecules.

3. *B. abortus* induces a type 1 cytokine profile from bovine, murine, and human T lymphocytes

Cytokines are key molecules that play a major role in determining a protective or noncurative immune response. Initially, $CD4^+$ T cells were divided into at least two subsets upon antigen stimulation. Currently, the type 1 or 2 cytokine concept has been expanded to other cell types such as $CD8^+$ T lymphocytes. The objective of this section is to compare the cytokine profiles induced by *B. abortus* in different animal species. The pattern of cytokines reported here was detected using RT-PCR for transcription profiles or ELISA bioassay for secreted products.

Brucella-primed bovine PBM cells showed an elevation of transcripts for IFN-γ but not for IL-2 or IL-4 when compared to PBM cells from naive animals (Covert and Splitter, unpublished data). An identical profile is exhibited by mouse splenocytes, and enriched $CD4^+$ and $CD8^+$ T lymphocytes (Oliveira, *et al.*, 1994a; Oliveira and Splitter, 1995; Svetic, *et al.*, 1993; Zhan, *et al.*, 1993; Zhan, *et al.*, 1995). In the murine system, the type 1 cytokine profile was confirmed by

detection of IFN-γ in the cell supernatants but not IL-2 or IL-4. A strong correlation between the level of cytokine mRNA transcripts and the cytokine pattern secreted by T lymphocytes was established, Table I. Interestingly, bovine and murine T cells exhibited a similar cytokine profile following stimulation with *B. abortus*, suggesting an analogous immune response between these two animal species and supporting the use of mice as a relevant model to study immunity to brucellosis.

B. abortus is a T-independent antigen and has been used as an antigen/carrier for different human vaccines (Blay, *et al.*, 1992; Zaiteva, *et al.*, 1995). In concordance with our studies, others have demonstrated that *B. abortus* induced IFN-γ secretion from purified human $CD4^+$ and $CD8^+$ T cells, revealing the ability of this bacterium to promote a type1 cytokine pattern of T cell differentiation. The similarity of the cytokine transcription profile as well as secreted products among bovine, murine and human T cells upon *B. abortus* stimulation is shown in Table I. Identical immune responses mounted by these different species against the same pathogen reinforces the use of the bovine system as an animal model to study human diseases and the applicability of immunological information among these species.

Table 1- *Brucella abortus* induces a type 1 cytokine response in bovine, murine and human T lymphocytes.

Species	Cell population	Cytokines						References
		mRNA			Protein			
		IL-2	IL-4	IFN-γ	IL-2	IL-4	IFN-γ	
Bovine	PBMC	-	-	+	N/D	N/D	N/D[a]	[b]
Mouse	splenocytes	-	-	+	-	-	+	[c]
	$CD4^+$	-	-	+	-	-	+	[d]
	$CD8^+$	-	-	+	N/D	N/D	N/D	[e]
Human	$CD4^+$	+	-	+	N/D	-	+	[f]
	$CD8^+$	-	-	+	N/D	-	+	[f]

[a] N/D, not done

[b] Covert and Splitter, unpublished
[c] Oliveira and Splitter, 1995; Zhan, *et al.*, 1993
[d] Oliveira, *et al.*, 1994a; Svetic, *et al.*, 1993; Zhan, *et al.*, 1995
[e] Oliveira and Splitter, 1995
[f] Blay, *et al.*, 1992; Zaiteva, *et al.*, 1995

4. The role of $CD4^+$ and $CD8^+$ T lymphocytes in resolution of disease

Previously, immunity to intracellular bacteria was considered to be exclusively mediated by $CD4^+$ T cells (Araya, *et al.*, 1989). Recently, optimal protection against intracellular bacteria has been regarded as a coordinated interaction between different T cell subsets (Ladel, 1994). Earlier murine brucellosis studies indicated the importance of T cell subsets in the resolution of infection but produced conflicting data suggesting either $CD4^+$ (Araya, *et al.*, 1989) and/or $CD8^+$ (Pavlov, *et al.*, 1989; Mielke, 1991) T cells were responsible for protection. The paradigm of disease resolution was founded on adoptive T cell transfer or *in vivo* subpopulation depletion using monoclonal antibodies; both with potential pitfalls (Dunn and North, 1991). To clarify the controversy over T cell subsets responsible for *B. abortus* disease resolution, we have used gene knockout mice. Mice in which the β2-microglobulin gene was disrupted or the MHC class II Aβ chain is nonfunctional (Koller and Smithies, 1989; Gosgrove,1991) are devoid of functional $CD8^+$ or $CD4^+$ αβ T cells, respectively. Using MHC class I and class II knockout (KO) mice and C57BL/6 mice as controls, $CD8^+$ T cells were found to be important in resolving *B. abortus* infection (Oliveira and Splitter, 1995). Moreover, our findings provide insight into both effector and regulatory mechanisms used by $CD8^+$ T cells to control an intracellular bacterial infection.

To determine the contribution of $CD4^+$ or $CD8^+$ T cells to controlling brucellosis, bacterial numbers were monitored in spleens of MHC class I KO, class II KO and C57BL/6 mice for four weeks following infection. The animals were sacrificed weekly and numbers of *B. abortus* colony forming units were determined. Interestingly, animals that lacked functional $CD8^+$ T cells (class I KO) had a marked increase in bacterial numbers when compared with class II KO or C57BL/6 control mice. After the first week post-infection, bacterial numbers increased in class I KO spleens, whereas a straight decline in bacterial colonies was noted in class II KO mice throughout the course of infection. Additionally, bacterial numbers in the spleens from class I KO mice were at least two logs higher than bacterial numbers in the class II deficient mice during the four weeks post-infection. To reinforce the importance of protection by $CD8^+$ T lymphocytes in gene knockout mice, $CD4^+$ T cells were depleted from class I KO and C57BL/6 mice, while $CD8^+$ T cells were depleted from class II KO and C57BL/6 mice. Depletion of $CD8^+$ T cells in class II KO and C57BL/6 mice caused bacterial numbers to increase. In contrast, depletion of $CD4^+$ T cells, by anti- L3T4 antibody treatment, in class I KO and C57BL/6 mice had no significant effect on bacterial numbers. Therefore, our observation that class I KO mice had enhanced susceptibility to brucellosis, while class II KO mice had rapid bacterial clearance, illustrates the impact of MHC I-dependent $CD8^+$ T cells in the acquisition of optimal resistance to *B. abortus* infection.

a. Cytokine analysis of splenocytes, $CD4^+$ and $CD8^+$ T lymphocytes

Cytokines are key molecules that play a major role in determining a protective or noncurative immune response; therefore, the cytokine transcription profile as well as secreted products were assayed. The cytokine mRNA pattern from infected and naive animals was determined by a competitive quantitative PCR assay. The results indicated that *Brucella* primed splenocytes from all three mice strains exhibited a type1 cytokine profile. Up-regulation of mRNA transcripts for IFN-γ but not IL-2 or IL-4 were detected. C57BL/6 or class I KO splenocytes displayed a ten-fold increase in the level of IFN-γ mRNA expression compared to splenocytes from naive animals. Interestingly, class II KO splenocytes had only a two-fold elevation of IFN-γ transcripts 2 weeks

following infection, but IFN-γ transcription reached levels similar to the other strains after this period. The lower IFN-γ expression observed in class II KO splenocytes early in infection likely resulted from the lack of $CD4^+$ T cells. ELISA assays for type 1 cytokines IL-2 and IFN-γ confirmed that IL-2 was not secreted when splenocytes from all three mice strains were cultured with γ-irradiated *B. abortus* or medium alone. However, cells from all mice strains secreted IFN-γ when cultured with γ-irradiated *B. abortus*. As expected, class II KO splenocytes produced less IFN-γ than cells from class I KO or C57BL/6. Thus, a strong correlation between the level of cytokine mRNA transcripts and the pattern of protein secreted by spleen cells was established. Substantial IL-10 activity was detected in spleen cell supernatants from all three mice strains 1 week post-infection. Class I KO spleen cells produced two-fold higher levels of IL-10 than splenocytes from class II KO or C57BL/6 mice.

In concordance with our study, others have also demonstrated that *B. abortus* strain 19 induced a novel cytokine transcription profile from murine $CD4^+$ T cells, featuring high levels of IFN-γ and IL-10 (Svetic, *et al.*, 1993). IL-10 has been identified as a cytokine that down-regulates protective immunity to *B. abortus* (Fernandes and Baldwin, 1995). IL-10 inhibits the anti-*Brucella* effector functions of macrophages as well as decreases the production by spleen cells of the protective cytokine IFN-γ following stimulation by *Brucella* antigens. We correlated the enhanced susceptibility of class I KO mice to brucellosis with the ability of class I KO spleen cells to produce higher levels of IL-10 (Oliveira and Splitter, 1995). The elevated IL-10 production by *Brucella*-activated macrophages may be associated with a high concentration of suppressor macrophages found in the spleens 2 to 3 weeks after infection with *B. abortus* (Cheers and Pagram, 1979). In summary, these findings indicate that *B. abortus* induces a type 1 subset response from murine $CD4^+$ and $CD8^+$ T cells, featuring high levels of IFN-γ and no elevation of IL-2 and IL-4 (Oliveira and Splitter, 1995; Oliveira, *et al.*, 1994a).

To better define the mechanisms used by $CD8^+$ T cells to control *B. abortus* infection, a cytokine transcription profile of this T cell subpopulation was determined. $CD8^+$ T lymphocytes from class II KO or C57BL/6 splenocytes were separated at 0 and 1 week after *B. abortus* infection using florescence activated cell sorting. Total RNA was extracted from sorted $CD8^+$ T cells and a competitive PCR was performed for IL-2, IL-4, IFN-γ and β-actin. $CD8^+$ T cells from class II KO or C57BL/6 mice had mRNA expression levels for IL-2 and IL-4 similar to $CD8^+$ T cells from untreated mice. However, IFN-γ mRNA was markedly elevated in $CD8^+$ T cells from both mice strains when compared with the naive group. In addition, primed $CD8^+$ T cells from class II KO mice expressed ten-fold higher transcripts for IFN-γ than $CD8^+$ T lymphocytes from C57BL/6 mice. The higher levels of IFN-γ expression observed in class II KO $CD8^+$ T cells might serve as a compensatory mechanism for the lack of $CD4^+$ T cells. Thus, *B. abortus* primed $CD8^+$ T cells from class II KO or C57BL/6 mice exhibited a type 1 cytokine profile identical to spleen cells from infected animals. IFN-γ is considered crucial for protection against *B. abortus* (Zhan, and Cheers, 1993; Baldwin, *et al.*, 1993). This cytokine is known to up-regulate macrophage microbial killing activity and induce development of Th1 cells (Kaufmann, 1993). Therefore, the production of IFN-γ by $CD8^+$ T cells seems to be one of the important mechanisms by which this T cell subpopulation helps the immune system to control infection. $CD8^+$ T cells are activated early in the immune response and are in an ideal position to regulate $CD4^+$ T cell development by producing IFN-γ or other regulatory cytokines. Moreover, we have determined that preactivated/memory $CD8^+$ cells

possessing a $CD44^{hi}$ $CD45RB^{lo}$ phenotype produce increased levels of IFN-γ compared with the naive cell group.

b. Cytotoxic activity of *B. abortus*-induced $CD8^+$ T cells

B. abortus specific $CD8^+$ T cells from C57BL/6 mice specifically lysed C57BL/6 infected splenic macrophages. Contrary to the expected, class II KO $CD8^+$ T cells failed to lyse class II KO infected macrophages. One explanation for minimal cytotoxic lymphocyte activity observed from class II KO $CD8^+$ T cells is the lack of MHC class II molecules, down-regulating expression of costimulatory molecules like B7 on antigen presenting cells that are critical for cell-cell activation (Nabavi, *et al.*, 1992). Preliminarily, we detected reduced expression of B7-1 and B7-2 on $Mac\text{-}1^+$ spleen cells from class II KO mice. Because the mutant mouse strain controls the infection as rapidly as C57BL/6 mice, killing infected target cells by cytotoxic $CD8^+$ T cells may not be one of the most critical arms of the immune system to combat brucellosis.

5. Immunization with recombinant bacterial proteins and genes

At present, live attenuated brucella are used to immunize domestic animals but no human vaccine is currently available. Therefore, developing an effective and safe vaccine for humans in geographic regions of high risk is important. One of our long term goals is to evaluate the immunization potential of genes encoding proteins that induce lymphocyte proliferation.

Efforts to understand and identify the bacterial antigens associated with activation of a protective cellular response have led to the isolation, cloning and characterization of several *B. abortus* proteins (Zhu, *et al.*, 1993; Oliveira, *et al.*, 1994b; Ficht, *et al.*,1986; Ficht, *et al.*, 1988; Ficht, *et al.*, 1990; Ficht,*et al.*, 1989; Bricker, *et al.*, 1990; Tibor, et al., 1994; Roop, *et al.*, 1994; Marquis, and Ficht, 1993; Roop, *et al.*, 1992; Gor, and Mayfield, 1992; Beck, et al., 1990). However, a single purified bacterial protein conferring a significant level of protection in animals has not been reported. Developing a defined vaccine with a well understood mechanism of action is critical for protecting army personnel.

Based on the historical evidence that ribosomal vaccines can protect individuals against bacterial infections, and the availability of this gene and its protein in recombinant form, we tested the central question of immune response and host protection. Mice were immunized using two different protocols. First, mice were immunized three times with recombinant L7/L12 fusion protein in adjuvant. Animals were challenged with 1×10^6 colony forming units of bacteria one week after the last immunization. The L7/L12 protein engendered a significant level of protection at 1, 2 and 4 weeks.

6. Macrophages *ex vivo* transfected with individual *B. abortus* genes

Recently, we have initially compared protection afforded by the L7L12 protein to other *B. abortus* proteins using macrophages transfected with individual brucella genes. The rationale for this approach is that transfected macrophages mimic protein processing and presentation to the immune system similar to natural infection. Macrophages were transfected with individual *Brucella* genes using the high-level eukaryotic expression vector pcDNA3, and the transfected cells selected using the neomycin analog G418. Mice Each group was immunized with 1×10^6 syngeneic macrophages transfected with one of 5 *Brucella* genes, i.e., *L7/L12*, *uvrA*, ssb, *GroEL*, *GroES*.

Control groups received cells transfected with the pcDNA3 vector containing the luciferase gene or vector only. On day 28 p.i., mice were challenged with $5x10^4$ CFU of *B. abortus* strain 2308. Mice were killed 14 days later, spleens removed, and the number of bacteria remaining determined by colony counts. Preliminary evidence using this approach indicates that mice immunized with *L7/L12* gene transfected macrophages have 1.64 logs of protection.

CONCLUSIONS

1. Using two methodologies, DNA probes based on amino acid sequence and lymphocytes to screen a bacterial library, we have described the isolation, molecular cloning and DNA sequence analysis of genes encoding *B. abortus* T lymphocyte reactive proteins.
2. Recombinant *Brucella* proteins produced from these identified genes stimulated primed $CD4^+$ lymphocytes to induce a type 1 cytokine profile.
3. Gene knockout mice deficient in $CD4^+$ or $CD8^+$ T cells indicated that $CD8^+$ T lymphocytes play a key role in reducing bacterial infection in animals. These $CD8^+$ T cells produce a type 1 cytokine profile characterized by high IFN-γ production, and that $CD8^+$ cells in normal mice possess cytotoxic activity against brucella infected macrophages.
4. Immunization of mice with recombinant L7/L12 protein conferred a significant level of protection.

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APPENDICES

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Isolation of *Brucella abortus ssb* and *uvrA* Genes from a Genomic Library by Use of Lymphocytes as Probes

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***Brucella abortus* proteins from virulent S2308 expressed from a pBluescript II SK⁻ genomic library stimulated peripheral blood mononuclear (PBM) cell proliferation from cattle vaccinated with *B. abortus* S19. The method described here permits a rapid and directed approach to isolate genes encoding antigens of *B. abortus* that interact with lymphocytes primed to the living bacterium. The supernatants from the bacterial host JM109 (DE3) were cultured with freshly isolated bovine PBM cells. A total of 300 clones were evaluated. Ten clones were identified that stimulated T-lymphocyte proliferation. Among them, one clone with a 2.5-kb insert stimulated T-lymphocyte proliferation in all three animals, suggesting that the proteins encoded by genes within this fragment may represent immunodominant antigens. DNA sequencing of this clone reveals two large open reading frames (ORFs). ORF II has a high degree of similarity to the *Escherichia coli ssb* gene, which codes for the single-stranded DNA binding protein. ORF I, in the opposite direction to ORF II, shows similarity to the N terminus of the *E. coli uvrA* gene, which codes for one of the three subunits of the *E. coli* ABC excision nuclease. The observation that the PBM cells recognized and proliferated in response to proteins expressed from single clones provides a novel strategy to select bacterial antigens that may prove useful in designing alternative vaccines against brucellosis.**

Brucella abortus is a facultative intracellular bacterium that causes febrile disease in humans and contagious abortion in many animal species (1, 39). Immunity to intracellular bacteria is mainly dependent on activation of the appropriate host cellular response (8, 13). Several lines of evidence suggest that cell-mediated immunity can limit the pathogenesis of brucellosis (4, 23, 34). *B. abortus* proteins that induce a T-lymphocyte-mediated response are most likely responsible for the observed protection. Therefore, identifying these bacterial proteins would be critical for understanding the immune response to *B. abortus* infection and/or vaccination.

Though T lymphocytes play a major role in protection against facultative intracellular bacteria, antigens relevant to the humoral immune response have been studied more extensively than T-cell antigens because antigens recognized by antibodies are more technically amenable to study (40). To identify antigens recognized by T lymphocytes, complex protein preparations from intracellular bacteria have been used to investigate the cellular immune response (12, 18, 37). However, isolation of individual stimulatory components in complex mixtures is still difficult. Alternative strategies of isolating genes that encode bacterial proteins which can be assayed for immunogenic potential have been tried (11, 33). Previously, a λgt11 *Mycobacterium leprae* DNA library was screened by using a pool of human CD4⁺ T-lymphocyte clones, and a DNA clone encoding an antigen that induced lymphocyte proliferation was isolated (20). But this approach requires considerable effort to derive T-cell clones and pools of recombinant antigens. Also, the antigens in the 96-well plates can be contaminated through the preparation process of multiple transfers.

In the present study, we screened a *B. abortus* genomic DNA library constructed in a pBluescript SK⁻ vector (Stratagene, La Jolla, Calif.) with bovine lymphocytes from animals primed to the living bacterium. The library has an average insertion size of 3 kb. Bulk-cultured lymphocytes instead of T-cell clones were used as probes representing the most heterogeneous T-lymphocyte repertoire. The bacterial proteins from 10 DNA clones, of 300 individual clones examined, were recognized by lymphocytes. One 2.5-kb clone stimulated T-lymphocyte proliferation in all three animals. DNA sequence analysis of this clone reveals two large open reading frames (ORFs) that have similarity to the *Escherichia coli ssb* gene (26) and the N terminus of the *uvrA* gene (15). This expedient approach to obtain proteins recognized by T lymphocytes provides a feasible method for the diagnosis and vaccine strategies required for brucellosis.

MATERIALS AND METHODS

Animals. Three Holstein cattle were kept at the University of Wisconsin Department of Animal Health and Biomedical Sciences. All three cattle received three injections of the attenuated live *B. abortus* S19 vaccine and were bled 1 year after the final vaccination. All animals lacked *B. abortus*-specific antibodies as detected by the *Brucella* card test (Hynson, Westcott and Dunning, Baltimore, Md.).

***B. abortus*.** *B. abortus* S19 was kindly provided by Barb Martin, Veterinary Services, National Animal Disease Center, Ames, Iowa. The bacilli were killed by gamma irradiation (^{137}Cs) and added to the adherent cells, and the culture was incubated overnight at 37°C as described elsewhere (32). Lymphocytes were added the following day for the proliferation assay.

***B. abortus* genomic library.** The library of *B. abortus* S2308, a virulent strain of *B. abortus*, was constructed in the *Hin*dIII restriction site of the pBluescript II SK⁻ phagemid.

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The library was the kind gift of Fred Tatum, National Animal Disease Center. The library contains about 80% recombinants with an average insertion size of 3 kb.

Preparation of antigens. The library was used to transform the competent JM109 (DE3) cells (Promega, Madison, Wisc.) according to the method of Hanahan (14). Then, 10 and 90% of the transformed bacteria were plated on LM plates (25) supplemented with 50 μg of ampicillin per ml. Colonies were allowed to grow overnight at 37°C, and single colonies were picked for further growth overnight at 37°C in 2 ml of SOB medium (25) containing 50 μg of ampicillin and 140 μg of isopropylthiogalactoside (IPTG) per ml. The saturated bacterial cultures were centrifuged at 1,500 × *g* for 30 min, and 30 μl of the supernatant was removed and added to the peripheral blood mononuclear (PBM) cells in 96-well plates.

Determination of antigen concentration. Previously, our laboratory isolated a T-lymphocyte-positive *B. abortus* clone (termed clone 12 kD) which was identified by using an oligonucleotide probe from the N-terminal sequence of a 12-kDa protein (6, 21). The clone was constructed by *Hin*dIII digestion of *B. abortus* chromosomal DNA to isolate the specific genomic fragment, which was then ligated into the *Hin*dIII site of pGEM-3Z (Promega). The clone was used to transform JM109 (DE3), and the antigen was prepared as stated above. Various concentrations of the supernatant, from 1 to 50 μl, were tested for the ability to induce lymphocyte proliferation, and the most stimulatory antigen concentration was determined.

Culturing of PBM cells. PBM cells were isolated from cattle blood by density gradient centrifugation by using Lymphoprep (Nycomed AS Diagnostics, Oslo, Norway). The cells were washed three times with phosphate-buffered saline containing 100 U of penicillin and 100 μg of streptomycin per ml. The PBM cells were then suspended in RPMI 1640 (Sigma, St. Louis, Mo.) supplemented with 2 mM L-glutamine–25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)–50 μM 2-mercaptoethanol–100 U of penicillin per ml–100 μg of streptomycin per ml–10% heat-inactivated fetal bovine serum (Sigma). The cells were cultured at a density of 10^6 cells per well in 96-well plates with the addition of 30 μl of prepared antigens or 10^7 whole killed *B. abortus* organisms at 37°C in a humidified 5% CO_2 atmosphere.

Proliferation assays. After 7 days of culture with killed *B. abortus* or *B. abortus* antigens, PBM cells were pulsed for the final 6 h with 1 μCi of [^{3}H]thymidine (Amersham Corp., Arlington Heights, Ill.) in each microwell. The cells were harvested onto glass fiber filters, and radioactivity was counted in a liquid scintillation counter. The experiments were performed two to four times.

Minipreparation of the phagemid DNA and restriction analysis. Minipreparation of DNA from clone 176 was done as described by Birnboim and Doly (5) with modifications by Johnson (16). The purified DNA was digested with the restriction enzyme *Hin*dIII (Bethesda Research Laboratories, Gaithersburg, Md.) according to the manufacturer's instructions. After digestion, the DNA was analyzed in a 0.75% agarose gel and stained with 0.5 μg of ethidium bromide per ml. The bands were identified with UV light.

DNA sequencing and data analysis. Both strands of the 2.5-kb double-stranded DNA fragments inserted into the pBluescript II SK$^-$ phagemid were sequenced by the chain termination method of Sanger et al. (27) with the Sequenase version 2.0 kit (United States Biochemical Corporation, Cleveland, Ohio). Synthetic oligonucleotide primers were used for both strands. An initial search of the GenEMBL data bank was done with the BLAST program (2). More detailed computer analysis of the DNA sequences was performed with the Genetics Computer Group sequence analysis software package (10). FASTA and TFASTA programs (22) were used to search the GenEMBL data bank.

Nucleotide sequence accession number. The *B. abortus* sequence data described here have been submitted to GenBank under accession number L10843.

RESULTS

Dose response to clone 12 kD by bovine PBM cells. Clone 12 kD, which stimulates lymphocyte proliferation, was tested to determine the appropriate amount of a *B. abortus* protein produced in JM109 (DE3). Various amounts of the supernatant from 1 to 50 μl were cultured with *B. abortus*-primed lymphocytes from one animal. Lymphocyte proliferation was observed with all concentrations of supernatant, but a plateau of proliferation was observed with 10 and 30 μl of supernatant per microwell, with 30 μl being optimal. Therefore, 30 μl of supernatant from protein-expressing phagemid clones was used to evaluate lymphocyte recognition of bacterial antigens.

Lymphocyte recognition of *B. abortus* proteins produced in the genomic library. The ability of *B. abortus*-primed PBM cells to recognize a variety of bacterial clones expressing *B. abortus* antigens was analyzed. Two hundred clones were examined for their ability to induce lymphocyte proliferation by using PBM cells from one animal. Supernatant from nontransformed JM109 (DE3) served as a negative control, and clone 12 kD served as a positive control. Clones that stimulated lymphocytes to proliferate fivefold more highly than the negative control were regarded as *B. abortus* antigens recognized by lymphocytes. Four positive clones (clones 176, 181, 182, and 193) were identified, as shown in Fig. 1A. These four positive clones were further tested in two other animals. Because the purpose of this study was to identify *B. abortus* proteins recognized by a number of animals, only proteins from clones recognized by several animals were further considered.

Responses to the four positive clones among three animals. To determine whether other animals could recognize the clones expressing *B. abortus* antigens, the four positive clones were further tested in two more animals. Only clone 176 was positive in all three animals (Fig. 2), indicating that certain *B. abortus* antigens are recognized by a number of animals while other antigens are more restricted with respect to cell-mediated recognition.

Additional *B. abortus* proteins from the genomic library recognized by lymphocytes from two animals. Clones 201 to 300 were tested in two animals. Five additional positive clones, 260, 289, 290, 292, and 298, were identified in both animals (Fig. 1B). Clone 262 was positive only in animal no. 154 (data not shown). Because animal 92 died during the experiment, the clones which were positive in animals 154 and 349 could not be further tested in that animal.

Lymphocyte response to clone 176, clone 12 kD, and killed *B. abortus*. The extent of lymphocyte proliferation in response to a *B. abortus* protein produced by the genomic library (clone 176) was compared with the proliferative response to a previously determined immunogenic protein (clone 12 kD) and gamma-irradiated *B. abortus*. Figure 3 illustrates that clone 176 stimulated bovine T-lymphocyte proliferation to an extent similar to that of the previously identified positive clone 12 kD. But the magnitude of lym-

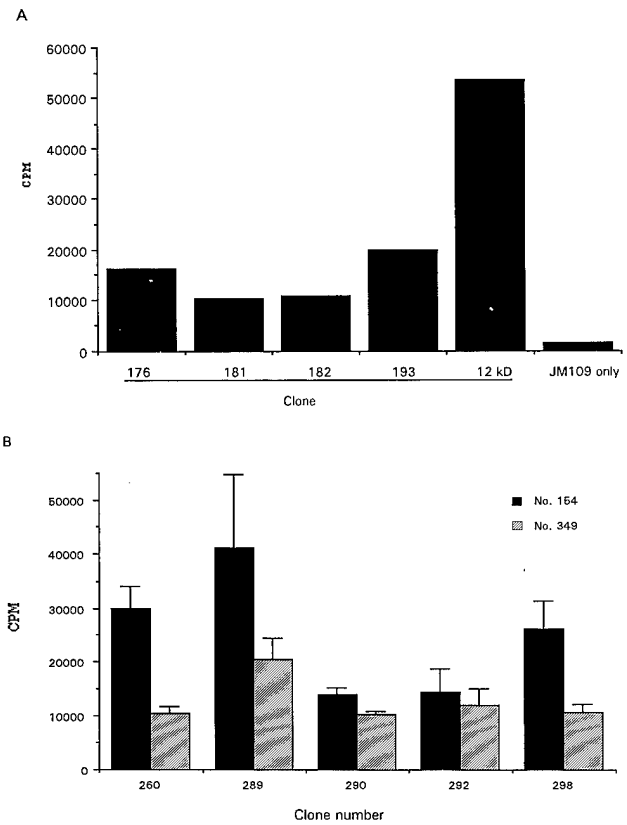


FIG. 1. T-cell proliferation in response to supernatants of positive bacterial clones. Antigens from the clones were prepared as described in Materials and Methods. A 30-μl volume of each antigen was added to microwells containing 10^6 PBM cells. The cells were then cultured for 7 days at 37°C for the proliferation assay, and lymphocyte cultures from animal 92 (A) and animals 154 and 349 (B) were counted. The response to JM109 (DE3) alone was less than 2,000 cpm for all animals. CPM, [^{3}H]thymidine incorporation, in counts per minute. Bars represent standard deviations.

phocyte proliferation in response to killed *B. abortus* was much greater than it was to the single protein expressed from clone 176. This result is likely due to the antigenic complexity of the whole organism compared with a single protein and the heterogeneity of the lymphocyte population. Clone 176 was selected as the first clone for additional molecular characterization. Restriction digestion of this clone with *Hin*dIII showed that the insertion is approximately 2.5 kb long (data not shown).

DNA sequence analysis. The 2.5-kb *Hin*dIII fragment was sequenced, and it revealed two large ORFs which are in opposite directions (Fig. 4A). Sequence analysis of both ORFs revealed putative −35 and −10 promoter regions as well as ribosome binding sites 5′ to the starting codon (Fig. 4B). The G+C content of the clone is 54%, similar to that of the *recA* gene of *B. abortus* (36). Both the nucleotide and the deduced amino acid sequences of ORF I are similar to the nucleotide and amino acid sequences of the N terminus of the *E. coli uvrA* gene and protein (79% similarity in 227 amino acids), which is a DNA repair enzyme that preferentially binds single-stranded or UV-irradiated double-stranded DNA (15). ORF II encodes 168 amino acids which share 70% similarity with *E. coli* single-stranded DNA-binding protein (26).

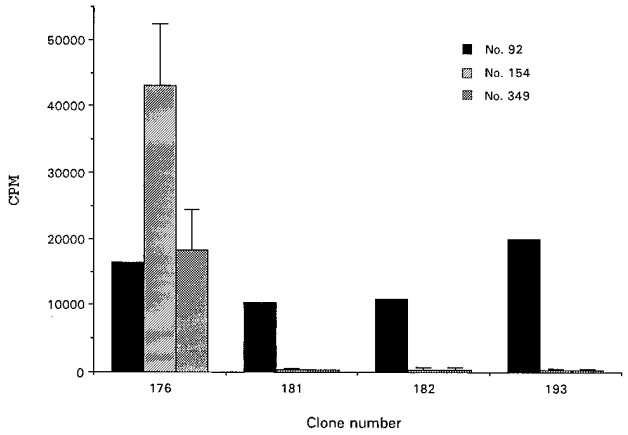


FIG. 2. Comparison of responses to clones 176, 181, 182, and 193 among three animals. A 30-μl sample of the antigen from each clone was added separately to 10^6 PBM cells from each of the three animals. After 7 days of incubation at 37°C, T-lymphocyte proliferation was assessed. The response to JM109 (DE3) alone was less than 2,000 cpm. CPM, [^{3}H]thymidine incorporation, in counts per minute. Bars represent standard deviations.

DISCUSSION

The results presented demonstrate a rapid and directed approach to isolate genes encoding proteins of *B. abortus* that interact with the bovine cellular immune system. By using a genomic library of a virulent strain of *B. abortus*, 10 clones were identified that encode proteins recognized by lymphocytes from animals vaccinated with live attenuated S19. Although the majority of these proteins induced lymphocyte proliferation in some but not all animals tested, one protein stimulated lymphocyte proliferation in all of the animals. Thus, proteins that induce antigen-specific lymphocyte proliferation in a number of animals, i.e., immunodominant proteins, can be recognized by use of this methodology. Although the importance of T lymphocytes specific for *B. abortus* has been recognized both in vivo (17) and in vitro (31) and although *B. abortus*-primed T lymphocytes can

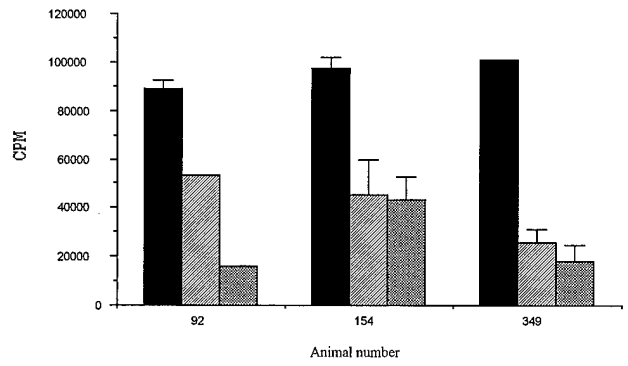
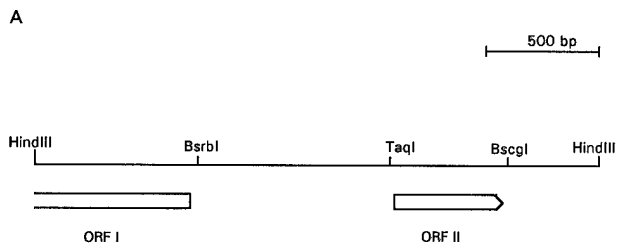


FIG. 3. Comparison of the lymphocyte proliferative responses to clone 176 (▩), clone 12 kD (▨), and killed *B. abortus* (■) among three animals. Either a 30-μl sample of the prepared antigen from clone 176 or clone 12 kD was added to the microwell containing the PBM cells or 10^7 killed *B. abortus* organisms were added to the adherent cells as described in Materials and Methods. The response to JM109 (DE3) alone was less than 2,000 cpm for the three animals. CPM, [^{3}H]thymidine incorporation, in counts per minute. Bars represent standard deviations.



B

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AG ACA GGT TTC GAG ACT GTC TGC AAG ACG TGT GGA CAA ATC CGG GCG CAC CAC CAC GCG GTC TAC   65
    C  T  E  L  S  D  A  L  R  T  S  L  D  P  R  V  V  V  R  D  V

CAC TAC GTC GAT ATC GTG CTT GTA TTT CTT GTC GAG TGG CGG AAC GTC GGC GAT CTC ATA GAA GGT  131
 V  V  D  I  D  H  K  Y  K  K  D  L  P  P  V  D  A  I  E  Y  F  T

GCC GTC CAC CTT CAC GCG CTG GAA GCC CTT TTT CTG AAG TTC GGC GAG TTC CTT CCT GTA TTC GCC  197
 G  D  V  K  V  R  Q  F  G  K  K  Q  L  E  A  L  E  K  R  Y  E  G

CTT ACG CCC GCG CAC GAT AGG CGC AAG GAT ATA AAG ACG TGT GCC TTC CTC CAG TGC GAT GAC CCT  263
 K  R  G  R  V  I  P  A  L  I  Y  L  R  T  G  E  E  L  A  I  V  R

ATC GAC CAT CTG GCT CAC CGT CTG GCT TTC GAT CGG AAG GCC GGT AGC AGG AGA ATA GGG AAT GCC  329
 D  V  M  Q  S  V  T  Q  S  E  I  P  L  G  T  A  P  S  Y  P  I  G

CAC ACG CGC AAA GAG CAG GCG CAT ATA GTC GTA GAT TTC GGT GAC TGT GCC CAC CGT CGA ACG CGG  395
 V  R  A  F  L  L  R  M  Y  D  Y  I  E  T  V  T  G  V  T  S  R  P

ATT GCG GCT TGT CGT CTT CTG CTC GAT GGA AAT GGC GGG CGA CAG GCC GTC AAT CTG GTC GAC ATC  461
 N  R  S  T  T  K  Q  E  I  S  I  A  P  S  L  G  D  I  Q  D  V  D

CGG TTT CTG CAT CAT TTC GAG AAA CTG ACG CGC ATA GGC TGA AAG GCT TTC GAC ATA GCG GCG CTG  527
 P  K  Q  M  M  E  L  F  Q  R  A  Y  A  S  L  S  E  V  Y  R  R  Q

GCC TTC GGC ATA GAT CGT ATC GAA AGC AAG CGA GGA TTT GCC CGA GCC AGA AAG CCC CGT CAT CAC  593
 G  E  A  Y  I  T  D  F  A  L  S  S  K  G  S  G  S  L  G  T  M  V

GAT CAG CTT GTC GCG CGG CAG ATC GAG ATC GAC ATT TTT CAG ATT GTG CTC GCG TGC GCC ACG TAT  659
 I  L  K  D  R  P  L  D  L  D  V  N  K  L  N  H  E  R  A  G  R  I
                                  SD
GGA AAT GAA TTT CTG ATC GCT CAT  TCCGCGTCCT GCCTGCTTCG TCCTGCCATG GATAACCGGG CGCCGAATGT  733
 S  I  F  K  Q  D  S  M
          -10                -35
GGGGATACGT CGCGCCGCTC CATTATATGG GGTGGACCTT CCAAGAAGAA GGGGTCTCCG GTCAATCGTC CGAGTGAACT  813

CATATCACCA GAACAAATAT CGAACAACCA GCCTTCTGAT ATGCGATACG AAATGTGAAC GCAAGAGCGT TGACTGTCAC  893

TCCTGACAGG AGCAGACTAC CCTTACTCGC CGATAGCGGA GAGATGCCGT GGGCGGAATA GCGCGGCATC GGCAAGAGGT  973

CGCAGGAGGC GGAAAACCCG GTAGGAGGTA AGGAGCATGA GTCCACCTGA CTAGGTTATG TCGCTTTCGT AAGAACGAAA 1053

GCACAATCGG ATTTGATTGT AGCGGCATCT TTTCGCAAGG AATGATGCAT CGTTGATCAA GATATCGATT GCCGAAAATT 1133

TACCTCACAC GCGGAAGGAA GATGGCTGTG CGCCATTGAT TTCCGAAAGA ATTTGTCGAC GAAGCACTGT CGACATGAGA 1213

GCTTTGCTCA ACCCCAAGCC CTGCGCGCCA AAGATACAGT GAGCGGCAGG GCTTTTCTTA TGGCTGTCCA GAGGGAAAAC 1293

CGGCCTTCGC GGCGTGCAAA GCGTAAAATG TCCTTAAAAT AGGGGAGAGA TGTTCATTTC GTTCACGCAA ATGCTTGCAT 1373

TTTGAACAAA ACAGGAACAT AACTCTGTGG ACATCAAAGA ATTCGCCTTC CAGGATTGAG CGCGTATTGA GCCAGTCCTG 1453
 -35        -10
TAAGGTGAAC ATTCTAGTTT CTGGTTATGG ACGCAGTTGC GCCTGACCTT TCTTTTGGAA AGCGCCCCTT GTCTGGACGA 1533
                    SD
AGACGTGTGG AAAAGGTGCG TGCAAGACCC GGAGTAAATT TTCG    ATG GCT GGT AGC GTC AAC AAG GTC ATT 1604
                                                   M  A  G  S  V  N  K  V  I

CTG GTC GGC AAT CTT GGT GCA GAT CCT GAA ATT CGC CGC CTG AAT TCC GGC GAT ATG GTT GCC AAC 1670
L  V  G  N  L  G  A  D  P  E  I  R  R  L  N  S  G  D  M  V  A  N

CTG CGC ATT GCA ACC TCG GAA AGC TGG CGT GAC CGC CAG ACC GGC GAA CGC AAG GAT CGC ACC GAA 1736
L  R  I  A  T  S  E  S  W  R  D  R  Q  T  G  E  R  K  D  R  T  E

TGG CAC AGC GTC GTC ATC TTC AAT GAA AAT CTC GCC AAG GTT GCC GAA CAA TAT CTG AAG AAG GGC 1802
W  H  S  V  V  I  F  N  E  N  L  A  K  V  A  E  Q  Y  L  K  K  G

GCC AAG GTT TAT ATC GAA GGC GCG CTC CAG ACC CGC AAG TGG CAG GAT CAA AAC GGC AAT GAC CGT 1868
A  K  V  Y  I  E  G  A  L  Q  T  R  K  W  Q  D  Q  N  G  N  D  R

TAT TCA AAG GAA ATC GTG CTG CAA AAA TTC CGT GGA GAG CTT CAA ATG CTC GAC AGC CGC AGC GAA 1934
Y  S  K  E  I  V  L  Q  K  F  R  G  E  L  Q  M  L  D  S  R  S  E

GGC GGC GAG GGC CGT TCC TTC GGC GGT GGT GGC AAC CGC AAC CAG ATG TCG GAT TAT TCC GGC GGC 2000
G  G  E  G  R  S  F  G  G  G  G  N  R  N  Q  M  S  D  Y  S  G  G

GGT GGA GAT TTC GGC TCA TCC GGC CCA TCT TCG GGC AGC AGC GGC GGT TTC TCG CGC GAT CTG GAC 2066
G  G  D  F  G  S  S  G  P  S  S  G  S  S  G  G  F  S  R  D  L  D

GAT GAA ATT CCG TTC TGA TCAGGTTTGA CGGGCGCTTC GGCCCTTTTC AGCAGCGTCT CTTTCGCATA TTTTCCGA 2142
D  E  I  P  F  *

AAGCTGCCCC CACTTTCGGG AGCGCGCTTC AGAGCCCATG GTCGGGCTGT CCTGATTTTC AGGGCTTTTT GACTGTATGT 2222

GCTCTGGTGT CTAAATAGGC TGAAATTCCC AGGGATCCTC TTGTCCAGTC ACGAAGTAGA CATAGGGGGC GTCTGACCGG 2302

AAAGGAACGT GGAGCCTGTC GTAAGCGAGA TTGTCGTGCC GTTATAAAGA AGAGGAAGAG CGGGCAGATA GTCAGACTTG 2382

GCGACCCAGG CAATAGTGGT GCCCACTGCT TATCAGGAGT ACCATTTCCA AGGCCAATAA GGGAGGCATC CAGTTCT    2459
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FIG. 4. Partial restriction map and sequence of the 2.5-kb fragment (clone 176). (A) Partial restriction map that shows the genes coding for UvrA (ORF I) and Ssb (ORF II) proteins of *B. abortus*. (B) Nucleotide sequence and deduced amino acid sequence for Ssb and the N terminus of the UvrA protein. Putative ribosome binding sites (SD) and promoter −10 and −35 regions are underlined. Presented here is the sequence of the minus strand of the *uvrA* gene, which is in the opposite direction to the *ssb* gene. The nucleotide sequence has been submitted to GenBank under the accession number L10843.

recognize specific *B. abortus* proteins (6) and extracted antigens from various *Brucella* species (7), few genes encoding *B. abortus* proteins recognized by lymphocytes have been reported (30). For the first time, we have used lymphocytes as probes to identify and isolate genes encoding immunodominant *B. abortus* proteins. These bacterial proteins involved in cell-mediated immune responses can facilitate our understanding of pathogenicity and host protection in brucellosis. The present attenuated *B. abortus* S19 vaccine has certain disadvantages, including aberrant serologic reactions (35), induction of disease in cattle (9) and humans (38), and failure of the vaccine (24). Because of these limitations, characterizing *B. abortus* antigens that activate lymphocytes provides insight into the mechanisms of vaccine-induced cell-mediated protection. Vaccine alternatives based on the strategy of selecting antigens that stimulate a strong lymphocyte response may be essential to resolve the current vaccine and diagnostic dilemma.

Our approach presented here also provides for the broadest cellular recognition of *B. abortus* antigens because bulk-cultured lymphocytes have been used. Using T-lymphocyte clones from single immune individuals as probes requires extensive lymphocyte cloning to identify a panel of proteins encoded by bacterial clones (19, 20). In contrast, bulk-cultured lymphocytes can rapidly and efficiently distinguish proteins encoded by bacterial clones that induce lymphocyte proliferation in a large number of individuals. Thus, antigens necessary for consideration as vaccines and diagnostic reagents can be quickly evaluated. The phenotypes of the bulk-cultured lymphocytes stimulated with previously identified *B. abortus* proteins, which included both $CD4^+$ and $CD8^+$ cells (6), were similar (data not shown). Therefore, use of bulk-cultured lymphocytes for the proliferation assay can indicate a T-lymphocyte response to *B. abortus* proteins.

In addition, the bulk-cultured lymphocytes used for the proliferation assays did not result in nonspecific reactivity to bacterial supernatants compared with reactivity to RPMI medium (Fig. 1A and data not shown). Others have reported a high level of nonspecific reactivity to crude phage lysates by bulk-cultured lymphocytes (19). The differences in the background responses may result from the different expression systems. *B. abortus* genes in the pBluescript vector were expressed in JM109 (DE3), and *E. coli* cells were then spun down to avoid any bacteria being added to the lymphocytes. The proteins in the supernatant, which could be released from dead bacteria after overnight culture, were added to the lymphocytes. The use of bovine instead of human lymphocytes might explain the low background response, since bovine lymphocytes do not proliferate in response to lipopolysaccharide of *E. coli* (data not shown) or *B. abortus* (3). Use of bulk-cultured lymphocytes provides a rapid approach to identifying genes encoding proteins recognized by T lymphocytes without the labor involved in maintaining T-cell clones.

Mustafa et al. (20) expected a 1/10,000 frequency of positive antigen signals from the λgt11 recombinants to induce the proliferation of T-cell clones. But minimal information has been reported on *B. abortus* antigens recognized by bulk-cultured lymphocytes. The number of *B. abortus* proteins recognized by whole T-cell populations from vaccinated cattle is uncertain. Previously, we have identified 38 *B. abortus* proteins that induced lymphocyte proliferation in 25 animals (6). Our initial strategy in the present study was to screen bacterial colonies until positive clones were identified. Then, we would test the positive clones in more than

one animal to determine the breadth of host recognition. Ten clones from the 300 recombinants stimulated bulk-cultured lymphocytes to proliferate from the vaccinated cattle. Future testing of these *B. abortus* cloned proteins as immunogens in nonvaccinated cattle will be necessary for evaluating them as potential vaccine candidates.

As proteins from clone 176 stimulated T-cell proliferation in all three animals, this clone was chosen for additional characterization by DNA sequencing. The nucleotide sequence of this 2.5-kb fragment revealed that it contains two large ORFs which encode two important bacterial DNA binding proteins, the Ssb and UvrA proteins. But as the vector contains both the T7 and *lac* promoters, it is not known whether Ssb and UvrA proteins are expressed from their own transcription-translation sequences or from the vector's promoters. Other studies have suggested that some bacterial gene promoters, e.g., those of mycobacteria, might be recognized by *E. coli* polymerases (28, 29). Similarly, *B. abortus* promoters can also be recognized by *E. coli* polymerases. It is obvious that this approach can only isolate the proteins whose translation sequences are recognized by the *E. coli* or fusion proteins which are in the correct reading frames. Both the *ssb* and *uvrA* genes discussed here contain the putative promoter sequences. Further studies are required to evaluate the use of these promoters in *B. abortus* and in *E. coli*. It was unknown at first whether the bovine PBM cells proliferated in response to the Ssb, UvrA, or fusion protein. Therefore both the *ssb* and *uvrA* genes were subcloned (data not shown), and the expressed proteins were added to the bovine PBM cells for inducing lymphocyte proliferation. The proliferation assay indicates that PBM cells proliferate in response to both the Ssb and UvrA proteins (data not shown). Purification of Ssb and UvrA will allow a more detailed examination of the immunodominant role that Ssb or UvrA protein plays in the mechanism of antigen recognition by bovine T lymphocytes.

In summary, our data show that individual *B. abortus* proteins from virulent S2308 can be identified from a genomic library by screening with bulk-cultured lymphocytes primed with S19 vaccine. Bacterial proteins expressed in the library can be recognized by a number of animals, suggesting their immunodominant nature. *B. abortus* Ssb or UvrA protein could be one of the major antigens recognized by bovine T lymphocytes. This approach raises the possibility that rapid isolation of a panel of bacterial antigens recognized by lymphocytes from different animals is feasible. Thus, potential antigen candidates from *B. abortus* that might serve as inducers of a protective cell-mediated immunity are possible.

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Brief Notes

Sequences of the *rplJL* operon containing the *L10* and *L7/L12* genes from *Brucella abortus**

(DNA sequencing; ribosomal proteins; bacterial genes)

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SUMMARY

The *rplJL* operon encodes the L10 and L7/L12 proteins, essential for ribosomal function and protein synthesis. In this study, we report the nucleotide sequence of the *rplJ* and *rplL* genes from *Brucella abortus*. The deduced amino-acid sequences show 37 and 67% identity to *Escherichia coli* L10 and L7/L12, respectively.

Brucella abortus is a facultative intracellular bacterium infecting animals and humans. This organism causes chronic infection and replicates within the cytoplasm of host mononuclear phagocytes. To understand the molecular mechanism used by *B. abortus* to penetrate and resist destruction within the macrophages, we need to identify and study genes and their encoded products related to critical functions for the organism, like protein synthesis and bacterial replication.

We report here the nt sequence of the *L10* and *L7/L12* genes that encode 50S r-proteins (Fig. 1). The L10 and L7/L12 proteins are essential in bacterial ribosomes for the proper function of factors like elongation factor-G (EF-G) and elongation factor-Tu (EF-Tu), involved in protein synthesis (Liljas and Gudkov, 1987). In *Escherichia coli* the genes encoding r-proteins L10 and L7/L12 are co-transcribed, and translation of both cistrons is regulated by binding of L10 or a complex of L10 and L7/L12 to a single target in the mRNA untranslated region that is located more than 100 nt upstream from the start codon (Climie and Friesen, 1988). Based upon sequence homology with the *E. coli* operon, we hypothesize that the same autogenous mechanism of translation regulation (feedback regulation) also takes place in the *B. abortus rplJL* operon.

L7/L12 is the most studied among the r-proteins and exists as a dimer, present in the *E. coli* ribosome in four copies. The L7/L12 protein is attached to the 23S RNA via the L10 protein. L7L12 consists of a N-terminal domain which is necessary for binding to L10 and a C-terminal domain which is important in the binding of factors like EF-Tu and EF-G. L7/L12 is essential in the ribosome for maximal rate and low error frequency of protein synthesis (Rice and Steitz, 1989).

L10 and *L7/L12* r-cluster genes were isolated from *B. abortus* (strain 19) genomic DNA using a degenerate synthetic oligo to the 5′ end of the *L7/L12* gene as a probe (Brooks-Worrel and Splitter, 1992). Among 38 *B. abortus* proteins analyzed by two-dimensional cellular immunoblotting, the L7/L12 was identified as the most

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*On request, the authors will supply detailed evidence for the conclusions reached in this Brief Note.

Abbreviations: aa, amino acid(s); *B.*, *Brucella*; BLAST, Basic Local Alignment Search Tool; bp, base pair(s); GCG, Genetic Computer Group (Madison, WI, USA); kb, kilobase(s) or 1000 bp; NCBI, National Center for Biotechnology Information; nt, nucleotide(s); oligo, oligodeoxyribonucleotide; psk⁻, pBluescript (Stratagene, La Jolla, CA, USA); r-, ribosomal.

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Fig. 1. Nucleotide sequence of the r-protein-encoding gene cluster from the *rplJL* operon. The predicted aa sequences are given below the first nt of each codon. The *L10* begins at nt 97 and the *L7/L12* gene begins at nt 766 (GenBank accession Nos. L19101 and L23505).

immunodominant protein to freshly isolated bovine lymphocytes (Brooks-Worrel and Splitter, 1992). The *B. abortus* genomic DNA was digested with *ClaI*, and the digested DNA was sized by agarose gel electrophoresis. Southern hybridization analysis, under low stringency conditions, was performed to identify the band containing both genes. The band was excised from the gel and the DNA purified using Geneclean (Bio 101, La Jolla, CA, USA). The isolated DNA fragment was then cloned into the *ClaI* site of the psk$^-$ phagemid. Next, the vector carrying the *L10* and *L7/L12* genes was used to transform *E. coli* XL1-blue strain to amplify the genes of interest. Plasmid DNA was extracted using Magic Miniprep (Promega, Madison, WI, USA) and both genes were sequenced using a Sequenase version 2.0 kit (US Biochemical, Cleveland, OH, USA) by the Sanger dideoxy-mediated chain-termination method (Sambrook et al., 1989). To complete the entire operon sequence internal oligo primers were made at the Biotechnology Center of University of Wisconsin (Madison, WI, USA).

The nt and deduced aa sequences of L10 and L7/L12 proteins are shown in the Fig. 1. The translation of the coding DNA was performed using a GCG sequence conversion program (Devereux et al., 1984). The *L10* gene is 516 nt (172 aa) long and the *L7/L12* is 372 nt (124 aa) long. Interestingly, not Met but Leu is the first aa of L10. The same putative start codon is found in the *L10* gene of citrus greening disease-associated bacterium that shares 50% identity with part of the deduced aa sequence of *B. abortus L10* gene (data not shown). The *B. abortus* L10 and L7/L12 share 37 and 67% identity with the equivalent deduced aa region of *E. coli* when the sequences of both genes were entered and searched in the GenBank using the BLAST network at the NCBI (Altschul et al., 1990).

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Subcloning and Expression of the *Brucella abortus L7/L12* Ribosomal Gene and T-Lymphocyte Recognition of the Recombinant Protein

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The *Brucella abortus L7/L12* ribosomal gene was amplified by PCR and subcloned into the prokaryotic expression vector pMAL-c2. *Escherichia coli* DH5α was transformed with the pMAL-L7/L12 construct, and gene expression was induced by IPTG (isopropyl-β-D-thiogalactopyranoside). The resulting fusion protein was purified by affinity chromatography and confirmed by Western blot (immunoblot) analysis using an anti-maltose-binding protein antibody. Additionally, purified recombinant L7/L12 protein induced T-lymphocyte proliferation of *B. abortus*-primed bovine peripheral blood mononuclear cells. Phenotypic analysis of the proliferating cell population demonstrated an increase in the percentage of $CD4^+$ T lymphocytes when peripheral blood mononuclear cells were cultured with recombinant L7/L12 compared with cells cultured in medium alone. Subcloning and expression of a *B. abortus* gene encoding a previously demonstrated immunodominant protein for bovine lymphocytes are important steps in selecting *Brucella* proteins that have potential as a component of a genetically engineered candidate vaccine.

Brucella abortus is a gram-negative intracellular bacterium infecting animals and humans (19). *B. abortus* infection causes spontaneous abortions in cattle and persistent undulant fever, endocarditis, arthritis, and meningitis in humans (27). Little is known regarding the cellular and molecular mechanisms used by *B. abortus* to penetrate and resist destruction within the macrophage, the principal host cell of residence for the bacterium. Gamma interferon is a cytokine produced by activated T lymphocytes and NK cells that plays an important role in antimicrobial activity through macrophage activation (1). This cytokine is capable of upregulating major pathways described for microbial killing by macrophages (4, 18, 24). Because *B. abortus* resides and replicates within the cytoplasm of mononuclear phagocytes and that cytokines such as gamma interferon upregulate macrophage killing activity, a T-lymphocyte-mediated response is crucial for host protection.

Few *B. abortus* genes have been cloned and the proteins that they encode have been characterized (6, 12, 15, 23, 29). Furthermore, little is understood regarding the importance of these bacterial proteins in the immune response. In addition, the potential for these proteins to elicit a protective cellular response against bovine brucellosis has not been investigated. Therefore, a greater understanding of the relationship of bacterial proteins with host protection and disease is required. Given that lymphocytes proliferate to bacterial antigens and that cytokines activate macrophage killing of intracellular bacteria, the identification of individual *B. abortus* proteins that trigger T lymphocytes is crucial to define how the immune system functions in this disease. At present, there is no information regarding recombinant *B. abortus* proteins that are able to activate a specific T-cell subset critical for providing host protection against this bacterium. This paper describes the subcloning of the *L7/L12* ribosomal gene from *B. abortus*, its expression in *Escherichia coli*, and the bovine T-lymphocyte recognition of the recombinant protein produced.

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Six cattle were kept at the Department of Animal Health and Biomedical Sciences, University of Wisconsin—Madison. Three animals received three injections of the attenuated live *B. abortus* vaccine and were the source of antigen-primed lymphocytes 1 year after the final vaccination. The other three animals were not vaccinated and served as negative controls.

The *B. abortus L7/L12* ribosomal gene was amplified by PCR and subcloned into the expression vector pMAL-c2 (New England Biolabs, Beverly, Mass.). Primers, containing one artificial restriction site at each end, were constructed according to the *L7/L12* nucleotide sequence (Genbank accession no. L19101 [21]). The primer sequences were (sense) 5′-CGCG GATCTAGAAAAATGGCTGATCTCGCAAAG-3′ (*Xba*I) and (antisense) 5′-GCGGGGCTGCAGCCAAACTTACTT GAGTTCAAC-3′ (*Pst*I). PCR was performed with a 100-μl volume containing 100 ng of DNA template (pBluescript II SK^- carrying the *L10* and *L7/L12* genes [21]), 1 μM of each primer, 2.5 mM $MgCl_2$, 200 μM (each) deoxynucleoside triphosphates, 1× PCR buffer, and 2.5 U of Ampli*Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.). PCR amplification was conducted with a DNA thermal cycler (Perkin-Elmer Cetus), using the following conditions: denaturation at 94°C for 90 s, annealing at 50°C for 90 s, and extension at 72°C for 150 s (25 cycles). The PCR-amplified product was purified by Geneclean (Bio 101, Inc., La Jolla, Calif.) and digested with *Xba*I and *Pst*I restriction endonucleases (GIBCO BRL, Gaithersburg, Md.). After digestion, the PCR product was purified again by the same procedure and ligated to the predigested pMAL-c2 vector, using DNA ligase (GIBCO BRL). The ligation reaction was used to transform *E. coli* DH5α, and single recombinant clones were selected. Plasmid DNA was extracted by Magic Miniprep (Promega Corp., Madison, Wis.) and digested by *Xba*I and *Pst*I to verify the presence of the insert. Positive *E. coli* clones possessing the pMAL-L7/L12 construct were selected, and expression of the fusion protein was induced by 0.6 mM IPTG (isopropyl-β-D-thiogalactopyranoside). After IPTG induction, bacterial cells were harvested by centrifugation at 4,000 × *g* for 20 min, and the supernatant

was discarded. The cell pellet was frozen in a dry ice-methanol bath at −70°C and allowed to thaw to 37°C. The pellet was resuspended in 100 ml of phosphate buffer saline (PBS; pH 8.4) containing 25 mg of lysozyme. The resuspended pellet was frozen and thawed three more times. The resulting suspension was homogenized to achieve uniformity and then incubated at 37°C for 30 min, with homogenization every 10 min. The suspension was centrifuged at 9,000 × *g* for 30 min, and the supernatant containing crude extract was loaded onto a polyacrylamide gel to confirm the presence of the expressed fusion protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). *E. coli* lysates containing the induced MBP-L7/L12 fusion protein were diluted 1:5 with PBS. Then, the suspension was loaded into an amylose resin column (New England Biolabs) and washed 10 times with PBS, and the fusion protein was eluted with PBS plus 10 mM maltose. The purified fusion protein was cleaved with factor Xa protease, which recognizes a specific amino acid sequence between the maltose-binding protein (MBP) and the L7/L12 protein. After cleavage, recombinant L7/L12 (rL7/L12) was purified by rebinding of MBP to the amylose resin. Pure rL7/L12 was concentrated in PBS, using a centricon-10 microconcentrator (AMICON, Beverly, Mass.), and the protein concentration was determined by a Bio-Rad (Hercules, Calif.) protein assay.

For SDS-PAGE lysates from induced and uninduced *E. coli* carrying the pMAL-L7/L12 construct, purified and cleaved MBP-L7/L12 fusion protein, pure MBP, and purified rL7/L12 were solubilized in sample buffer containing 2% SDS and 5% 2-mercaptoethanol and processed according to the method of Laemmli (10). Before electrophoresis an equal volume of 2× concentrated sample buffer was added, and the mixture was boiled for 3 min and loaded onto the gel. Protein samples and molecular weight markers were analyzed on SDS–15% PAGE gels and visualized by Coomassie blue staining.

For Western blot (immunoblot) analysis, the gels were electroblotted onto nitrocellulose at 75 V for 2 h. Transfers were carried out in 25 mM Tris–192 mM glycine–20% methanol (3, 26). The blotted nitrocellulose was blocked with skim milk for 2 h. Then, rabbit anti-MBP serum was used at a 1:5,000 dilution during incubation for 2 h (at room temperature). After reactions with the primary antibody, the blots were washed three times with TBST (0.5 M NaCl–0.02 M Tris [pH 7.5], 0.05% Tween 20) and incubated for 1 h with a goat anti-rabbit immunoglobulin G–alkaline phosphatase conjugate (Promega) at a 1:10,000 dilution in TBST. Then, the blots were washed three times with TBST, and the reactions were developed by using nitroblue tetrazolium and BCIP (5-bromo-4-chloro-3-indolyl-1-phosphate) purchased from Promega.

To express the *B. abortus L7/L12* gene in *E. coli*, PCR amplification of the target gene was done with further subcloning of *L7/L12* into pMAL-c2. This vector expresses foreign sequences as a fusion protein with the 42.7-kDa MBP (8). Expression of a 55-kDa fusion protein was observed by SDS-PAGE containing lysates from IPTG-induced *E. coli* that contained the pMAL-L7/L12 construct (Fig. 1A). To confirm that the expressed fusion protein was MBP-L7/L12, Western blot analysis was performed, using rabbit anti-MBP antibody. Figure 1B shows the anti-MBP recognition of the MBP-L7/L12 fusion protein. After expression, the fusion protein was purified in an amylose affinity column, and the interval necessary for cleavage of L7/L12 from MBP was determined by kinetic experiments at 2, 4, 8, 14 h with factor Xa treatment. Incubation of factor Xa protease with the fusion protein for 14 h resulted in total cleavage of MBP from L7/L12 (Fig. 2). Cleavage of the fusion protein shown in Fig. 2 confirmed the expected molecular masses of MBP and L7/L12, 42.7 and 12 kDa, respectively. rL7/L12 was purified by rebinding MBP to the amylose resin. Figure 3A shows the isolated rL7/L12 on an SDS-PAGE after purification. Western blot analysis demonstrated that the anti-MBP antibody did not recognize the separated rL7/L12 (Fig. 3B).

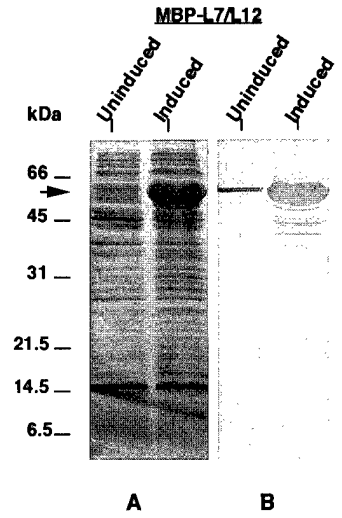

FIG. 1. SDS-PAGE profile and corresponding Western blot analysis of the recombinant MBP-L7/L12 fusion protein. (A) Coomassie blue-stained SDS–15% PAGE of lysates from uninduced and induced *E. coli* expressing the pMAL-L7/L12 construct. (B) Immunoblot of the recombinant MBP-L7/L12 fusion protein probed with rabbit anti-MBP antibody.

Peripheral blood mononuclear (PBM) cells were isolated from cattle blood samples by density gradient centrifugation with Lymphoprep (Nycomed AS Diagnostics, Olso, Norway). The cells were washed three times with PBS containing 100 U of penicillin per ml and 100 μg of streptomycin per ml. The PBM cells were then suspended in RPMI 1640 (Sigma Chemical Co., St. Louis, Mo.) supplemented with 2 mM L-glutamine, 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), 50 μM 2-mercaptoethanol, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 10% heat-inactivated fetal bovine serum (Sigma). Isolated PBM cells were cultured for 4 days at a density of 5×10^5 cells per well in 96-well plates

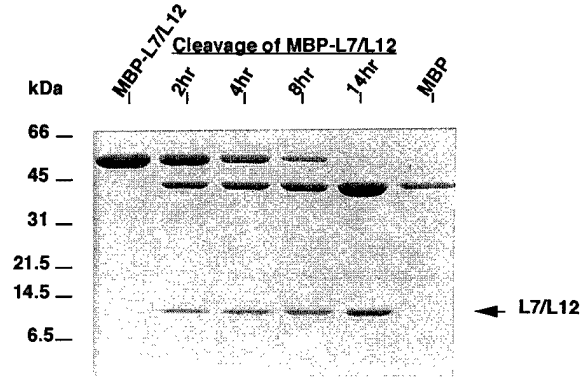

FIG. 2. SDS-PAGE analysis of MBP-L7/L12 fusion protein cleavage by treatment with factor Xa protease at 2, 4, 8, and 14 h. Pure MBP and noncleaved MBP-L7/L12 were used as controls. Molecular mass markers are shown on the left, and pure L7/L12, released from MBP, is indicated by the arrow on the right.

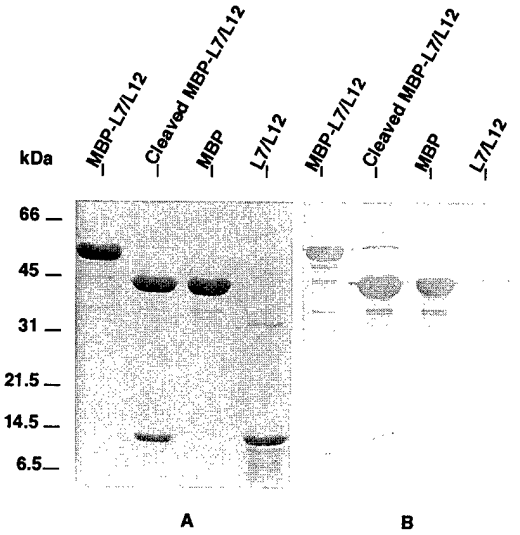

FIG. 3. (A) Coomassie blue-stained SDS–15% PAGE profile of MBP-L7/L12 fusion protein, cleaved MBP-L7/L12, pure MBP, and purified L7/L12 ribosomal protein. (B) Electrotransferred samples from SDS-PAGE were analyzed by immunoblotting with rabbit anti-MBP antibody.

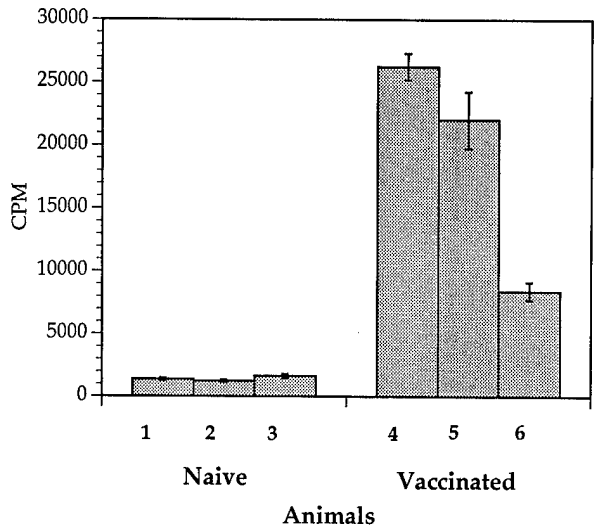

FIG. 4. Proliferative response of PBM cells from *B. abortus*-vaccinated cattle and naive animals. PBM cells were stimulated with 50 μg of rL7/L12 or concanavalin A per ml. Concanavalin A-stimulated cells from both groups of animals had responses of >100,000 cpm (data not shown). Results are expressed as mean cpm. Error bars indicate standard errors of the means.

with the addition of 50 μg of rL7/L12 (optimal concentration was determined after testing 1 to 100 μg/ml) or concanavalin A (2.5 μg/ml) as a T-cell-activating control in a final volume of 200 μl per well. After this culture period, lymphocytes were pulsed for the final 18 h with 1 μCi of [^{3}H]thymidine (Amersham Corp., Arlington Heights, Ill.) in each well. The cells were harvested onto glass fiber filters, and the radioactivity was determined in a liquid scintillation counter. Assays were performed in triplicate.

Primed bovine PBM cells from three different animals proliferated to the rL7/L12 protein, whereas cells from the naive group did not (Fig. 4). Concanavalin A was used as a T-cell-activating control in this experiment, and the counts from both the naive and vaccinated group were above 100,000 cpm (data not shown). The lymphocyte population engaged in this response was analyzed by flow cytometry. Phenotypic surface analysis was conducted on proliferating cells 7 days following stimulation with the rL7/L12. To analyze the membrane phenotypic markers, PBM cells (10^6) in 0.1 ml of PBS containing 1% bovine serum albumin (Sigma) and 0.05 ml of an anti-bovine surface marker monoclonal antibody were incubated on ice for 30 min. The cells were washed twice with PBS prior to suspension in 50 μl (0.5 μg) of anti-mouse immunoglobulin-dichlorotriazinylaminofluorescein (Ig-DTAF; H & L Jackson Laboratories, Avondale, Pa.). The cells were incubated in the dark for 30 min on ice, washed twice, suspended in PBS, and analyzed by flow cytometry (Becton Dickinson, Mountain View, Calif.). The monoclonal antibodies used were MM1A (anti-CD3 [5]), IL-A12 (anti-CD4 [2]), SBU-T8 (anti-CD8 [14]), 86D (anti-γδ T-cell receptor [13]), and 33 (anti-bovine IgM [20]). The cells that proliferated in response to the recombinant protein (their approximate percentages ± standard deviations are given in parentheses) were $CD4^+$ (20.7% ± 0.8%), $CD8^+$ (13.2% ± 1.6%), IgM (10.7% ± 2.1%), and γδ-T-cell receptor (4.5% ± 1.0%). We detected a twofold increase in the percentage of $CD4^+$ T lymphocytes when PBM cells were stimulated in vitro with the rL7/L12 (20.7% ± 0.8%) compared with cells cultured in medium alone (10.8% ± 1.5%). However, no significant change in the percentage of the other cell types was detected (data not shown).

In this study, we have subcloned and expressed a *B. abortus* DNA fragment which encodes the L7/L12 ribosomal protein previously shown to be immunodominant in cattle (3). The *L7/L12* gene encodes a 50S ribosomal protein and is essential in bacterial ribosomes for the proper function of factors involved in protein synthesis, such as elongation factor G and elongation factor Tu (11). L7/L12 consists of an N-terminal domain that anchors the L10 ribosomal protein and a C-terminal domain which is important in the binding of elongation factors G and Tu. The L7/L12 ribosomal protein is essential for the maximal rate of protein synthesis and the low frequency of errors (22).

The in vivo prokaryotic protein expression system used here produced large amounts of the recombinant fusion polypeptide and proved to be a suitable system for expressing foreign sequences and purifying the resultant fusion protein. The pMAL-c2 vector has been used for gene expression of other immunodominant proteins, such as the GroES heat shock protein from *Mycobacterium leprae* (16). Having manufactured the recombinant protein, we demonstrated that L7/L12 is able to specifically stimulate PBM cells from *B. abortus*-primed animals. Phenotypic analysis of the proliferating cells demonstrated that the percentage of $CD4^+$ T cells was twofold higher when PBM cells were stimulated with rL7/L12 than with medium alone. Other investigators have recently confirmed the immunogenic nature of an analogous protein, when they reported the L7/L12 ribosomal protein from *Mycobacterium bovis* as a strong delayed-type hypersensitivity stimulus for sensitized guinea pigs (25). Furthermore, inflammatory reactions including the delayed-type hypersensitivity response in brucellosis and listeriosis have been shown to be mediated exclusively by $CD4^+$ T cells (17).

Ribosomal preparations from several pathogens, including *B. abortus*, have been shown to be highly protective vaccines (7). However, the components of these preparations have not

yet been characterized. A *Salmonella typhimurium* ribosomal vaccine afforded cell-mediated protection, based on the capacity of primed T cells to respond to *Salmonella* antigens and to activate macrophages (9). The mechanism by which the host responds to and eliminates infection is a central issue in our understanding of the immune response to pathogens. For many intracellular organisms, such as *B. abortus*, T-cell-mediated immunity is critical for host protection. Because T cells play a major role in protection against *Brucella* infection through cytokine secretion, such as that of gamma interferon (17), the identification of specific *Brucella* antigens that induce a T-lymphocyte response is an important element in designing new molecular candidate vaccines. Soluble *Brucella* proteins have been isolated and tested for their ability to stimulate primed murine T lymphocytes by cell proliferation and cytokine production (28). These fractions induced T-lymphocyte proliferation and elicited gamma interferon production by $CD4^+$ T cells, but none of the proteins have been purified and characterized, suggesting that a number of proteins rather than a single moiety possess these antigenic determinants.

This is the first report of a *B. abortus* T-cell-reactive ribosomal protein. However, further characterization of the T-lymphocyte subset involved in this response by phenotypic surface analysis and cytokine profile is required. Identifying proteins from a given pathogen that favor a T-cell response and the desired cytokine profile may provide important approaches for diagnosis and alternative vaccine strategies. Subcloning and expression of the *B. abortus L7/L12* gene provides an opportunity to immunologically characterize a specific ribosomal protein which may clarify why ribosomal vaccines confer protection against intracellular pathogens.

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Recombinant L7/L12 ribosomal protein and γ-irradiated *Brucella abortus* induce a T-helper 1 subset response from murine CD4$^+$ T cells

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SUMMARY

Immunity to *Brucella abortus* crucially depends on antigen (Ag)-specific T-cell mediated activation of macrophages, which are the major effectors of cell-mediated killing of this organism. Ribosomal preparations have been used as vaccines against several pathogens, including *B. abortus*, conferring a high degree of protection. In the present study, we have examined the pattern of T-helper (Th) cell response from infected BALB/c mice after *in vitro* stimulation with recombinant (r) L7/L12 ribosomal protein or γ-irradiated *B. abortus*. In addition to Ag-specific proliferation, CD4$^+$ T cells were tested for interleukin-2 (IL-2), IL-4 and interferon-γ (IFN-γ) mRNA expression and secretion. Detection of cytokine transcripts and secreted cytokines was performed using reverse transcriptase (RT)–polymerase chain reaction (PCR) and specific ELISA assays. Primed CD4$^+$ T cells proliferated to the recombinant protein or whole *B. abortus*. The functional cytokine profile of the proliferating cells was typical of a Th1 cell phenotype, as we detected transcripts for IL-2 and IFN-γ but not IL-4. Among the cytokines analysed, only IFN-γ produced in the Th cell culture supernatants was detected by ELISA when bacteria or recombinant protein were used. Thus, rL7/L12 ribosomal protein and γ-irradiated *B. abortus* preferentially stimulated IFN-γ-producing Th1 cells after *in vitro* stimulation. The results of this study provide for the first time an explanation of why ribosomal vaccines may protect against intracellular infections, and an experimental basis for identifying polypeptides from a pathogen which stimulates the desired cytokine profile and Th cell response crucial for the design of genetically engineered candidate vaccines.

INTRODUCTION

Brucella abortus is a facultative intracellular bacterium that infects humans and domestic animals.[1] *Brucella* replicates in host mononuclear phagocytes, and survival in phagocytic cells allows the bacterium to escape the extracellular mechanisms of host response such as complement and antibodies.[2] The pathological manifestations of brucellosis are diverse and include arthritis, endocarditis and meningitis in humans, while bovine brucellosis is characterized by abortion.[3]

Immunity to *B. abortus* involves T-cell dependent activation of macrophages, the main cellular reservoir for the bacterium.[4] CD4$^+$ T cells play an important role in protection against *Brucella* infection, either by activating CD8$^+$ T cells or secreting cytokines that mediate macrophage activation.[5] Even though the cytokine network involved in brucellosis is not completely understood, interferon-γ (IFN-γ) does play a prominent function in up-regulation of macrophage anti-*Brucella* activity, which presumably requires activation of the CD4$^+$ T-helper 1 (Th1) subset.[4] The ability of specific antigen (Ag) to induce preferentially a Th1 or Th2 subset response is an important aspect for the development of molecular vaccines against intracellular pathogens.[6] These distinct T-cell subsets often influence the outcome of infection through the production of specific cytokines.[7] *Brucella abortus* induces a Th1 cytokine pattern from human T cells, and the bacterium has been considered as a potential carrier for human immunodeficiency virus (HIV) and malaria vaccines.[8,9] Th1 subset engagement in response to *Brucella* proteins appears to be a decisive cell-mediated mechanism to provide resistance against *B. abortus* infection through IFN-γ production. Injection of IFN-γ into mice enhanced resistance to *B. abortus*, while injection of anti-IFN-γ monoclonal antibody (mAb) exacerbated the infection.[4] Therefore, the identification of specific *Brucella* Ag that preferentially induce a Th1 subset response provides a viable, focused approach to design new molecular candidate vaccines.

Among 38 *Brucella* proteins recognized by lymphocytes from primed animals, we have identified the L7/L12 ribosomal protein as an immunodominant Ag from this bacterium.[10,11] The immunological importance of ribosomal preparations from 28 different pathogens, including *B. abortus*, has been studied by several investigators.[12–15] These ribosomal Ag

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confer a high degree of protection when used as vaccines. However, the components of these preparations have not yet been characterized. Recently, the L7/L12 ribosomal protein from *Mycobacterium bovis* was identified as a strong delayed-type hypersensitivity (DTH) stimulus for sensitized guinea-pigs,[16] which heightened the interest in determining the importance of ribosomal Ag in protective acquired immunity. We have previously demonstrated that peripheral blood mononuclear cells (PBMC) from cattle primed with live *B. abortus* recognized and proliferated to the recombinant (r) L7/L12 ribosomal protein (S. C. Oliveira, unpublished observations). Therefore, the present study was designed to characterize the nature of the Th cell response to rL7/L12 and, based on the pattern of cytokines produced, evaluate the potential of rL7/L12 as a component of a genetically engineered vaccine. This study for the first time characterizes immunologically a recombinant Th cell reactive protein from *B. abortus* and may provide an explanation as to why ribosomal vaccines protect against intracellular pathogens.

MATERIALS AND METHODS

Mice

Female BALB/c mice were purchased at 5–8 weeks of age from Harlan Sprague–Dawley (Indianapolis, IN) and were used for experimental infection. All animals were housed in a Biosafety Level (BL)-3 facility and handled according to University of Wisconsin-Madison Research Animal Resource Center guidelines.

Bacteria

Brucella abortus, the live attenuated vaccine strain 19, was obtained from Dr Barbara Martin (National Animal Disease Center, Ames, IA) at 2×10^{10} colony-forming units (CFU)/ml. For use as Ag in T-cell cultures, the bacteria were killed by γ-irradiation with ^{137}Cs (250 000 rads).

Infection

BALB/c mice received an intravenous (i.v.) injection of 5×10^4 CFU *B. abortus* in 100 μl of sterile phosphate-buffered saline (PBS). Eight weeks postinfection, when the number of *Brucella* CFU was diminished in the spleen, the animals were killed by cervical dislocation and the spleen harvested for T-cell preparation.

Expression and purification of the rL7/L12

For expression of the *L7/L12* ribosomal gene and purification of the protein produced, the pMAL-c2 expression vector system (New England Biolabs, Beverly, MA) was chosen. Briefly, the *L7/L12* gene was inserted downstream from the *malE* gene of *Escherichia coli*, which encodes the maltose-binding protein (MBP), resulting in the expression of a MBP–L7/L12 fusion protein.[17] *Escherichia coli* (DH5α strain) expression of the *L7/L12* gene was induced by 0·6 mM isopropyl-β-thiogalactopyranoside (IPTG). The bacterial cells were lysed by freezing/thawing plus lysozyme treatment and centrifuged at 9000 ***g*** for 30 min. The supernatant was diluted 1 : 5 in PBS and loaded in an amylose column to affinity purify the fusion protein. The pMAL-c2 vector also contains the sequence coding for the recognition site of the specific protease factor Xa, which allows MBP to be cleaved from the polypeptide of interest after fusion protein purification. Factor Xa cleavage was carried out at a (w/w) ratio of 1% of the amount of fusion protein, and incubated at room temperature for 14 hr. After cleavage, the rL7/L12 was purified by rebinding the MBP to the amylose column. The purified rL7/L12 protein was concentrated in PBS using a centricon-10 microconcentrator (Amicon Inc., Beverly, MA), and the protein concentration determined by BioRad protein assay (Biorad, Hercules, CA) according to manufacturer's directions.

SDS–PAGE

For SDS–PAGE, *E. coli* lysates carrying the MBP–L7/L12 construct, purified MBP–L7/L12 fusion protein, MBP only and purified rL7/L12 were solubilized in sample buffer containing 2% SDS and 5% 2-mercaptoethanol (ME) and processed as described previously.[10] Before electrophoresis, an equal volume of 2 × concentrated sample buffer was added, the mixture boiled for 3 min, and loaded onto the gel. Proteins were analysed on a 15% SDS–PAGE gel and visualized by Coomassie staining.

Purification of $CD4^+$ T cells and flow cytometric analysis

Single-cell suspensions were prepared from the spleen of infected and naive animals. Splenocytes were isolated by density gradient centrifugation using lympholyte-M (Cedarlane Laboratories, Ontario, Canada). Cells were washed three times with sterile PBS containing 100 U/ml of penicillin, 100 μg/ml of streptomycin and 50 μg/ml of gentamicin and resuspended in ice-cold RPMI-1640 (Sigma Chemical Co., St Louis, MO) supplemented with 2 mM L-glutamine, 100 U/ml of penicillin, 100 μg/ml of streptomycin, 25 mM HEPES, 5×10^{-2} M 2-ME and 10% heat-inactivated fetal bovine serum (Sigma). $CD4^+$ T cells were purified using an affinity chromatography column (Biotec Laboratories Inc., Alberta, Canada) by a process of negative selection with rat anti-mouse Ly2 (CD8a) mAb and polyclonal goat anti-mouse IgG, according to the manufacturer's directions. The T-cell phenotype of the purified splenocytes was confirmed by flow cytometry using a panel of mAb. Goat anti-mouse immunoglobulin (catalogue no. 6300), anti-mouse L3T4 (GK1.5), anti-mouse Thy-1.2 (30-H12) and anti-mouse Lyt-2 (53-6.7) were purchased from Becton Dickinson (San Jose, CA). Anti-mouse $\gamma\delta$ T-cell receptor (TCR; GL3), and anti-mouse natural killer (NK) cells (PK136) were obtained from PharMingen (San Diego, CA). Briefly, purified $CD4^+$ T cells (5×10^5) were incubated for 30 min on ice with the panel of labelled mAb listed above. After extensive washing the cells were resuspended in PBS and further analysed by flow cytometry. Monoclonal antibodies of similar isotype but specific for an unrelated Ag were used as negative controls. Flow cytometric analysis confirmed that >92% of the negatively selected splenocytes expressed CD4 molecules on their surface. In the $CD4^+$-enriched population, $CD8^+$, $\gamma\delta$ T cells and NK cells, which represent potential IFN-γ producers, were <1% for each cell type.

T-cell proliferation assay

Murine $CD4^+$ T cells (1×10^5) from naive and infected animals were stimulated with 50 μg/ml of rL7/L12 (the optimal concentration was determined after testing 1–100 μg/ml), with

1×10^7 γ-irradiated CFU *B. abortus* or concanavalin A (Con A; 2·5 μg/ml) as a T-cell-activating control, in a final volume of 200 μl/well in 96-well plates. Syngeneic irradiated (2000 rads) spleen cells (3×10^5) from naive animals were added as antigen-presenting cells (APC) to the $CD4^+$ T cells. The cells were cultured for 4 days and pulsed with [^{3}H]thymidine ([^{3}H]TdR; 1 μCi/well) for 18 hr. Cells were harvested and scintillation counting performed. Assays were performed in triplicate.

Cytokine analysis

Following the same protocol used to stimulate $CD4^+$ T cells in the T-cell proliferation assay, cell cultures were monitored for cytokine gene expression and production at 2, 4, 6 and 10 days after Ag stimulation using cytokine-specific ELISA assays and reverse transcriptase (RT)–polymerase chain reaction (PCR) analysis. RT-PCR was used to detect β-actin, interleukin-2 (IL-2), IL-4 and IFN-γ mRNA expression. Briefly, cultured $CD4^+$ T cells (5×10^5) were washed with fresh RPMI and total RNA was extracted from the cells using guanidinum thiocyanate (TRI reagent, Molecular Research Center Inc., Cincinnati, OH). Total RNA (2 μg) was subjected to first-strand cDNA synthesis in a 20-μl reaction containing 1·5 mM of each dNTP, 2 μg oligo dT_{15}, 3 μg of acetylated BSA, 1 × RT buffer, 40 U RNasin (Promega Corporation, Madison, WI) and 400 U moloney murine leukaemia virus RT (Gibco BRL, Gaithersburg, MD), for 1–2 hr at 37°. After completion of first-strand synthesis, the specific cDNA template was amplified by PCR. PCR reactions contained, in a 50-μl volume, 3 μl of the RT reaction as template, 2·5 mM $MgCl_2$, 200 μM of each dNTP, 1 μM of each specific primer, buffer as supplied by the manufacturer and 2·5 U *Amplitaq* DNA polymerase (Perkin-Elmer, Norwalk, CT).[18] The β-actin, IL-2, IL-4 and IFN-γ primers used for amplification during the PCR reaction were obtained from Stratagene (La Jolla, CA) and the predicted product sizes were 245, 451, 279, and 405 bp, respectively. PCR reactions were performed using a 480-DNA thermal cycler (Perkin-Elmer, Norwalk, CT) in the following conditions: denaturation at 94° for 30 min, annealing at 60° for 5 min followed by 35 cycles of 1·5 min at 72°, 45 seconds at 94° and 45 seconds at 60°, with a final extension of 10 min at 72°. To detect the amplified products, 6 μl of the final reaction mix was run on a 2% agarose gel with molecular weight (MW) standards at 100 V for 1·5 hr. The prestained gels with ethidium bromide (0·5 μg/ml) were visualized under UV light and photographed. Cytokines were detected in the supernatant of the $CD4^+$ T-cell cultures using ELISA to mouse IFN-γ (Genzyme, Cambridge, MA) IL-2 and IL-4 (Endogen, Boston, MA). The assays were performed in duplicate according to the manufacturer's directions.[19,20]

RESULTS

Expression of *B. abortus* L7/L12 ribosomal gene in *E. coli*

To obtain large amounts of the rL7/L12-encoded polypeptide, the gene was expressed in *E. coli* using the pMAL-c2 expression vector system. *Escherichia coli* harbouring the pMAL-L7/L12 construct produced optimal amounts of a 56 000 MW fusion protein that permitted purification by affinity chromatography. Following cleavage of the fusion protein by factor Xa protease,

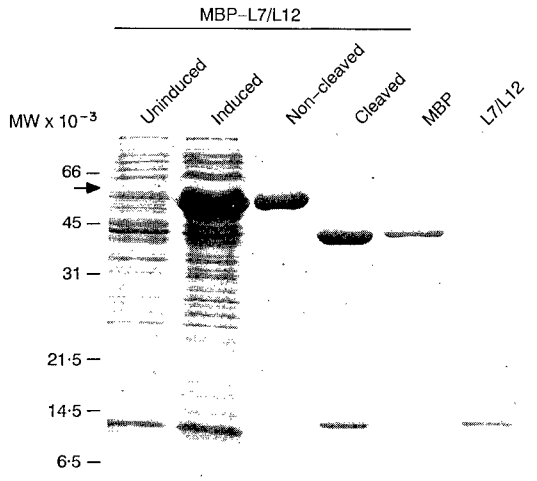

Figure 1. SDS–PAGE containing expression products of the pMAL–L7/L12 construct. The uninduced and induced lanes show lysates from *E. coli* harbouring pMAL–L7/L12. The non-cleaved lane shows affinity-purified fusion protein (56 000 MW). The cleaved lane shows the fusion protein cleaved by the factor Xa protease, showing MBP (42 700 MW) and rL7/L12 (13 000 MW). The MBP lane shows the pure MBP used as control, and the L7/L12 lane shows the purified recombinant ribosomal protein after factor Xa cleavage.

which recognizes a motif between the MBP and L7/L12, the resulting products were analysed on a 15% SDS–PAGE gel (Fig. 1). After cleavage the rL7/L12 protein was purified from the MBP and used as Ag in T-cell stimulation and cytokine assays.

$CD4^+$ T-cell proliferative response

$CD4^+$ T cells were isolated and purified from spleens of mice that had been infected with live *B. abortus* strain 19, and the Ag-specific T-cell response was compared with the response of cells from naive animals. $CD4^+$ T cells from primed animals responded vigorously to whole γ-irradiated *B. abortus*, as well as the rL7/L12, when these preparations were used as Ag (Fig. 2). However, when $CD4^+$ T cells from naive mice were

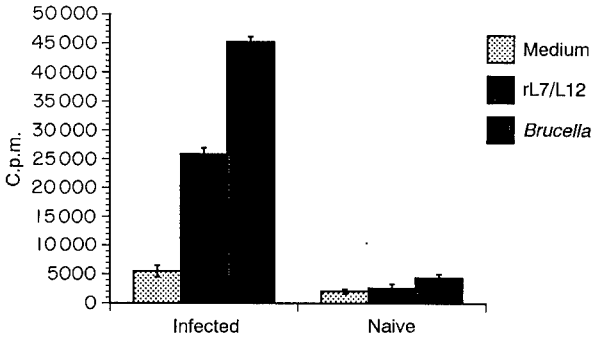

Figure 2. Proliferative responses of $CD4^+$ T-cell cultures derived from *B. abortus*-infected and naive BALB/c mice. Th cells were stimulated with rL7/L12, γ-irradiated *B. abortus* or Con A. Con A-stimulated cells from infected and naive animals had c.p.m. counts above 100 000 (S. C. Oliveira, unpublished observations). Results are expressed in c.p.m. ± SE.

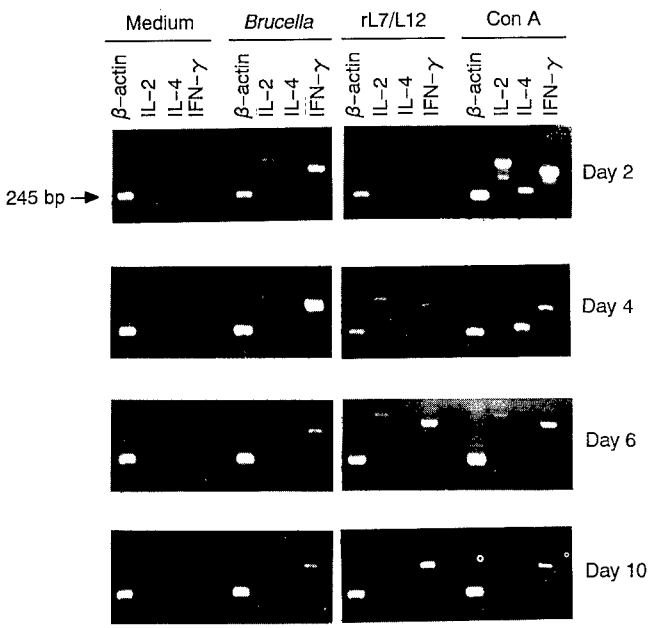

Figure 3. Pattern of β-actin, IL-2, IL-4 and IFN-γ mRNA expression in $CD4^+$ T cells from *B. abortus*-infected mice and mice stimulated *in vitro* with rL7/L12, γ-irradiated *B. abortus* or Con A. At days 2, 4, 6 and 10 of culture, total RNA was extracted, and the mRNA expressed was determined by RT-PCR. Transcripts from $CD4^+$ T cells cultured with medium alone were analysed as a negative control.

used no significant T-cell proliferative response was detectable. Taken together, these results indicate that Th cells from infected mice respond specifically to stimulation with the rL7/L12 or whole *B. abortus*. Furthermore, we have demonstrated that PBMC from *B. abortus*-primed cattle are able to respond to the rL7/L12 *in vitro* (S. C. Oliveira, unpublished observations), demonstrating that T-cell recognition of this particular Ag is not species specific.

Analysis of cytokine-specific mRNA

The cytokine mRNA pattern was determined by RT-PCR following stimulation of Th cells with rL7/L12 or whole *B. abortus*. The results of the mRNA analyses for a panel of cytokines demonstrated that when primed $CD4^+$ T cells were stimulated with either rL7/L12 or whole *B. abortus*, IL-2 and IFN-γ mRNA expression was detected, particularly on days 2 and 4 after stimulation (Fig. 3). Specifically, IL-2 expression was detected on days 2 and 4 in *B. abortus* and days 2, 4 and 6 in rL7/L12 cultures. IFN-γ mRNA was observed with the bacterium or recombinant protein at all intervals tested. Expression of IL-4 transcript, a hallmark of the Th2 subset, was undetectable when either whole *B. abortus* or rL7/L12 was used as Ag. However, when Con A was used as a positive control, all cytokines tested were detected. Only β-actin mRNA was observed when medium alone was used. This cytokine transcription profile indicated that rL7/L12 and whole *B. abortus* preferentially induced a Th1 subset response.

rL7/L12 and whole *B. abortus* preferentially induced IFN-γ-producing Th1 type cells

To confirm that cytokines were secreted as well as transcribed from Ag-activated Th cells, ELISA assays for IL-2, IL-4 and

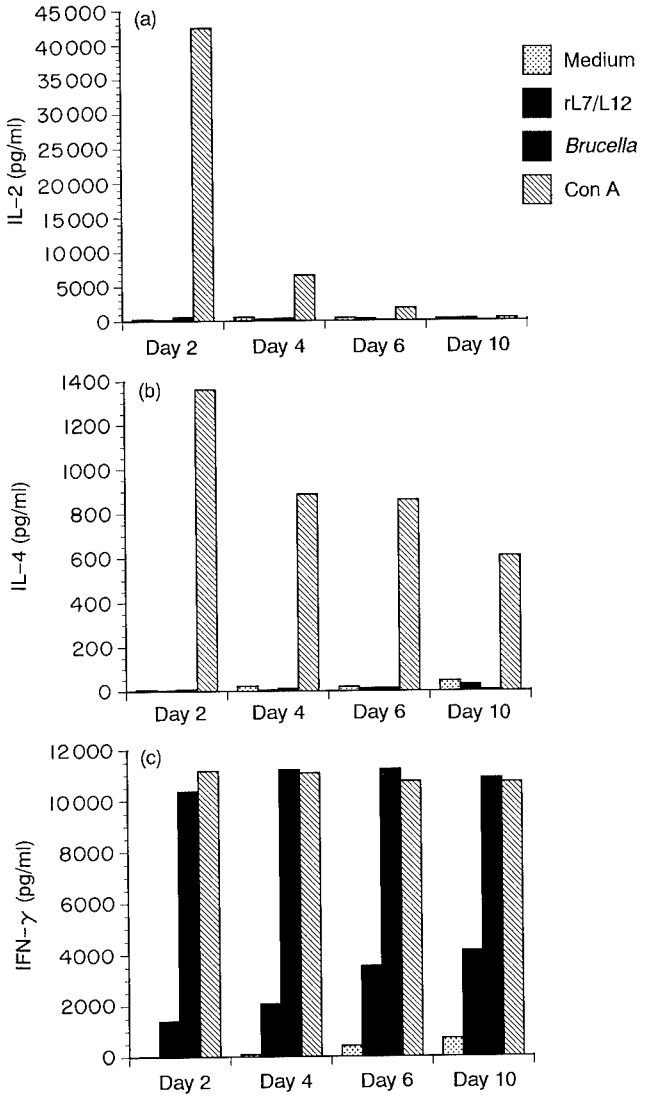

Figure 4. Cytokine responses of $CD4^+$ T cells from *B. abortus*-infected BALB/c mice and mice stimulated *in vitro* with rL7/L12, γ-irradiated *B. abortus* or Con A. ELISA for IL-2 (a), IL-4 (b) and IFN-γ (c) present in cell culture supernatants at days 2, 4, 6 and 10 are shown. Supernatant of cells cultured with medium alone were tested as a negative control.

IFN-γ were performed. A $CD4^+$ T-cell cytokine response to the rL7/L12 or whole *B. abortus* was demonstrable for IFN-γ but not for IL-2 and IL-4 (Fig. 4a, b and c). Whole *B. abortus* was a strong stimulus for IFN-γ production, reaching similar levels to Con A induction. On the other hand, IL-2, a typical Th1 cytokine, was not evident in the Th cell culture supernatants when either bacteria or recombinant protein were used. Additionally, rL7/L12 induced significant production of IFN-γ, a cytokine of central importance to enhance resistance to intracellular pathogens such as *B. abortus*.[21–23] These results demonstrated that whole *B. abortus* and rL7/L12 preferentially induced IFN-γ-producing Th1 cells. Taken together, these data also provided a strong correlation between the presence of transcripts for the cytokines analysed and the pattern of protein secreted in the Th cell culture supernatants. IL-4 mRNA and secreted protein were not detectable in $CD4^+$ T cells stimulated

with rL7/L12 or whole *B. abortus*. In contrast, IFN-γ transcript was detected from days 2 to 10 and accompanied by cytokine secretion during the same interval. Interestingly, IL-2 mRNA was observed in our kinetics experiments early, 2 and 4 days post-stimulation, whereas cytokine secretion was undetectable by ELISA. We hypothesize that small amounts of IL-2 produced was sequestered by soluble IL-2 receptors (IL-2R) and/or expanded Th cells expressing high-affinity IL-2R.[24,25]

DISCUSSION

In these experiments, we have characterized a recombinant Th1 cell-reactive protein from *B. abortus*. The rL7/L12 ribosomal protein and whole γ-irradiated *B. abortus* preferentially stimulated a specific $CD4^+$ Th1 type of response in a murine model. These findings were achieved by *in vitro* detection of secreted cytokines using specific ELISA assays, and cytokine mRNA transcription profiles of the stimulated Th cells using RT-PCR. Purified $CD4^+$ T cells were used to ensure that the Ag effects noted were due to direct stimulation of T cells and not to stimulation of other contaminating cell types that could influence the cytokine pattern observed. The use of T-cell clones was avoided given that long-term culture with exogenous cytokines in splenocyte cultures may have regulatory effects on the *in vitro* development of $CD4^+$ Th1 or Th2 clones.[26] Our results indicate that, after stimulation with the rL7/L12 or whole *B. abortus*, $CD4^+$ T cells expressed transcripts for IL-2 and IFN-γ, and secreted IFN-γ, into the culture supernatants. Human PBMC, when stimulated with heat-inactivated *B. abortus*, also expressed IL-2 and IFN-γ mRNA and secreted IFN-γ as measured by ELISA.[8,9] We have confirmed recent findings that spleen cells from mice infected with heat-killed *B. abortus* produce significant amounts of IFN-γ in culture supernatants and no measurable IL-2 during the first 4 weeks following infection, as measured by bioassays.[27] In contrast, our data differ from others who have suggested that γ-irradiated *B. abortus* has the ability to suppress IFN-γ production by bovine PBMC.[28] The detection of IL-2 mRNA by RT-PCR in our experiments probably reflects trace amounts of IL-2 mRNA expressed above constitutive levels. However, the ELISA assay confirmed no IL-2 secretion in the $CD4^+$ T-cell culture supernatant. The absence of detectable IL-2 in the culture supernatant is consistent with the previous finding that reported no significant increase in IL-2 gene expression in mice splenocytes following immunization with *B. abortus*.[29] These previous investigators suggested that IL-2 may not play a major role in the host immune response against *B. abortus*, and implied that IFN-γ gene expression appears to be IL-2 independent. The rL7/L12 ribosomal protein previously shown to be an immunodominant protein in cattle[10] induced murine $CD4^+$ T cells to express and secrete a pattern of cytokines similar to the profile induced by whole *B. abortus*, which is typical of a Th1 subset response. Identifying similar immune responses and lymphokine profiles produced to individual proteins compared to the whole organism may provide important approaches for diagnosis and alternative vaccine strategies.

In mice infected with *Leishmania major*[30,31] as well as *B. abortus*,[29] IFN-γ has been shown to regulate Th1 and Th2 cells by inhibiting the proliferative response of the Th2 subset. Therefore, IFN-γ is an important factor influencing which type of Th cell predominates after infection and, consequently, the outcome of disease.[32] Resistance to tuberculosis and brucellosis crucially depends on antigen-specific T-cell mediated activation of macrophages, which are the major effectors of cell-mediated killing.[2,33] Ag that preferentially induce an IFN-γ-producing Th1 subset response are desirable subunits of any vaccine preparation against brucellosis.

Additionally, a ribosomal vaccine prepared from *Salmonella typhimurium* afforded cell-mediated protection, based on the capacity of primed T cells to respond to *Salmonella* Ag, and correlated with the degree of macrophage activation engendered by this immunization.[34] Other investigators have commercially produced an intranasal polyvalent ribosomal vaccine against human respiratory disease, which has exhibited a high degree of success.[35] Because of the efficacy of ribosomal vaccines against different pathogens[12] and the possible contamination of these preparations with other cellular Ag, it is desirable to identify the specific antigenic determinant(s) responsible for protection.

High levels of IFN-γ produced by Th cells after whole *B. abortus* stimulation may be one reason why the commercially available *B. abortus* strain 19 live vaccine confers protection to domestic animals. However, this live preparation is pathogenic for humans,[1] causes abortion when administered to pregnant cattle[36] and, lastly, the vaccine can induce antibodies in vaccinated animals that interfere with the diagnosis of field infection.[37] Therefore, characterizing the immune response to a bacterial ribosomal protein previously shown to be immunodominant[10] is a feasible approach to designing molecularly based subunit vaccines and overcoming the problems comprising the currently available vaccine.

In summary, our findings provide an explanation to the question why ribosomal preparations from several different pathogens protect against infection, including tuberculosis and brucellosis.[16,38] Others had associated the potent adjuvant property of contaminating rRNA with the ability of ribosomal vaccines to confer protection.[39] However, additional studies have revealed that treatment of ribosomes with ribonuclease did not affect the immunogenicity of some ribosomal preparations, whereas treatment with proteolytic enzymes had abrogated the immunogenic nature of such Ag.[40] In this study, we have produced a recombinant Th cell-reactive ribosomal protein and characterized its importance immunologically. Additionally, we have shown that proteins from a given pathogen can be identified that favour the desired cytokine profile and Th cell response important for the design of molecular candidate vaccines.

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Expression of *tfx* and Sensitivity to the Rhizobial Peptide Antibiotic Trifolitoxin in a Taxonomically Distinct Group of α-Proteobacteria Including the Animal Pathogen *Brucella abortus*

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Three phylogenetically distinct groups within the α-proteobacteria which differ in trifolitoxin sensitivity are described. Trifolitoxin sensitivity was found in strains of *Agrobacterium*, *Brucella*, *Mycoplana*, *Ochrobactrum*, *Phyllobacterium*, *Rhodobacter*, *Rhodopseudomonas*, *Rhodospirillum*, and *Rhizobium*. Strains of *Agrobacterium*, *Brucella*, *Phyllobacterium*, *Rhizobium*, and *Rhodospirillum* were capable of producing trifolitoxin upon conjugal transfer of *tfxABCDEFG*.

Triplett and Barta (24) found that trifolitoxin (TFX), a posttranslationally modified peptide antibiotic, inhibited only strains of *Rhizobium leguminosarum* and *Rhizobium fredii*. Bradyrhizobia, plant pathogens, and enteric bacteria were not inhibited by TFX. By comparison of the sequences of 16S rRNA genes and other analyses, a number of very interesting bacteria have been found to be close relatives of the genus *Rhizobium* (10, 16, 29–32). These include members of the leaf-nodulating genus *Phylobacterium* (14); intracellular animal pathogens such as *Bartonella*, *Brucella*, and *Rochalimaea* spp. (4, 6, 17, 18, 26); soilborne and aquatic diazotrophs such as *Beijerinckia*, *Blastobacter*, and *Mycoplana* spp.; *Ochrobactrum anthropi*, a symbiont of nematodes (1) which can cause bacteremia in humans (11); and photosynthetic diazotrophs such as *Rhodobacter*, *Rhodospirillum*, and *Rhodopseudomonas* spp.

In this work, the TFX sensitivities of species within the α-proteobacteria were determined. The construction, replicons, inserts, and references of the plasmids used in this work are listed in Table 1. DNA isolations and manipulations were primarily performed as described by Sambrook et al. (19). Bacterial strains and culture media used in this work are included in Table 2. Antibiotic concentrations used were described previously (8) except that the tetracycline concentration used was 1.5 μg/ml. *Brucella* strains were cultured on *Brucella* agar medium (0964-01-3; Difco) at 37°C with 5% CO_2 or at 28°C. All other α-proteobacteria were cultured at 28°C.

A modification of the triparental mating procedure described by Triplett et al. (25) was used here in the conjugation of all plasmids into α-proteobacteria. Cells were taken directly from stocks frozen at −70°C in 15% glycerol except *Escherichia coli* strains and *Brucella abortus* RB51 (20), which were

TABLE 1. Plasmids used in testing TFX sensitivity and in the transfer of *tfx* to various α-proteobacteria

Plasmid	Replicon (reference) and insertion site	Deletion or gene(s) added to replicon	Antibiotic resistance phenotype[a]	Reference
pTFX3[b]	pTFX2 (25); *Hpa*I deletion	Deletion of internal *Hpa*I fragment of Tn*5*	**Tc^r**	This work
pTFX23	pBluescript II KS$^+$ (2); *Hin*cII	7.1-kb *Mlu*I fragment of pTFX1 (22) containing *tfxABCDEFG*	Ap^r	8
pTFX24	pBluescript II KS$^+$ (2); *Hin*cII	Same as pTFX23 but in opposite orientation	Ap^r	8
pTFX24K	pTFX24 (8); *Xho*I	Insertion of 1.2-kb *Hin*cII fragment from pUX-BF5 (5) containing *npt*II	Ap^r Km^r	This work
pTX24K	pTFX24K; *Not*I deletion	Deletion of 1.5-kb *Not*I fragment containing *tfxAB'*	Ap^r Km^r	This work
pDTXC-12	pDSK519 (12); *Eco*RI, *Sst*I	5.8-kb *Not*I fragment of pTFX23 containing *tfxB'CDEFG*	**Km^r** TFX^r	This work
pDT42[b]	pDSK519 (12); *Eco*RI, *Sst*I	7.2-kb *Bss*HII fragment of pTFX24 containing *tfxABCDEFG*	**Km^r** TFX^r	This work
pT2TX3K	pTR102 (28); *Bam*HI	6.8-kb *Bss*HII fragment of pTX24K containing *tfxB'CDEFG*	Ap^r **Km^r Tc^r** TFX^r	This work
pT2TFXK[b]	pTR102 (28); *Bam*HI	8.3-kb *Bss*HII fragment of pTFX24K containing *tfxABCDEFG* and *npt*II	Ap^r **Km^r Tc^r** TFX^r	This work

[a] Antibiotic resistances in bold were used to select against recipients in triparental matings with α-proteobacteria.
[b] Plasmid confers TFX production in *Rhizobium* spp. upon transfer by conjugation.

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TABLE 2. TFX sensitivities of selected α-proteobacteria

Organism	Growth inhibition (% of control)[a] by TFX from:	
	T24	KIM5s(pT2TFXK)
Agrobacterium radiobacter		
ATCC 19358[b]	0	0
C58[b]	0	0
Agrobacterium rhizogenes ATCC 11325[b]	75	109
Agrobacterium rubi TR3[b]	2	2
Agrobacterium vitis		
CG-48[b]	13	19
CG-64[b]	208	22
Azorhizobium caulinodans ATCC 43989[b]	0	0
Beijerinckia indica ATCC 9037[b]	0	0
Blastobacter denitrificans ATCC 43295[b]	0	0
Bradyrhizobium elkanii 61A76[b]	0	0
Bradyrhizobium japonicum 110Spc[b]	0	0
Mycoplana dimorpha ATCC 4279[b]	74	118
Ochrobactrum anthropi		
ATCC 49188[b]	30	52
ATCC 49188[c]	0	23
Phyllobacterium myrsinacearum		
ATCC 43590[b]	0	0
ATCC 43590[c]	5	23
Phyllobacterium rubiacearum		
ATCC 43591[b]	4	32
ATCC 43591[c]	16	58
Rhizobium etli		
USDA9032[b]	91	133
USDA9041[b]	167	158
USDA9043[b]	135	164
Rhizobium galegae		
USDA4128[b]	0	0
USDA4130[b]	13	9
USDA4136[b]	0	8
Rhizobium huakuii		
USDA4773[b]	9	39
USDA4776[b]	0	4
USDA4778[b]	0	0
Rhizobium leguminosarum 128C1[d]	100	100
Rhizobium meliloti		
102F3[b]	3	15
1021[b]	0	0
Rhizobium tropici		
USDA9039[b]	0	0
USDA9030[b]	0	30
Rhizobium sp. strain ANU280[b]	45	64
Rhodobacter capsulatus B10[e]	0	0
Rhodobacter sphaeroides 2.4.1[e]	68	78
Rhodopseudomonas marina BN126[e]	18	35
Rhodopseudomonas palustris RPI[e]	0	0
Rhodospirillum rubrum UR2[e]	19	28

[a] Values are percentages of the area of the inhibition zone obtained with *R. leguminosarum* bv. viceae 128C1. Sources of TFX production were the producing strains T24 and KIM5s(pT2TFXK). No zones of inhibition were observed with the nonproducing strains T24::Tn*5*$_1$ and KIM5s(pT2TX3K) (data not shown).

[b] BSM (7) was used to culture test strains and the TFX-producing strains.

[c] YM (27) medium was used to culture test strains and the TFX-producing strains.

[d] 128C1 was cultured on the same medium as the test strains.

[e] ATCC medium 1139 was used to culture test strains and the TFX-producing strains.

TABLE 3. TFX production by transconjugants of *Agrobacterium*, *Brucella*, *Phyllobacterium*, *Rhizobium*, and *Rhodospirillum* spp.[a]

Species and transconjugant[b]	Area of net inhibition zone (mm^2)	
	128C1	102F3
Agrobacterium rhizogenes		
ATCC 11325(pDT42)	2,312	69
ATCC 11325(pDTXC-12)	0	0
Brucella abortus		
RB51(pDT42)	1,979	0
RB51(pDTXC-12)	0	0
Phyllobacterium rubiacearum		
ATCC 43591(pDT42)	1,477	59
ATCC 43591(pDTXC-12)	0	0
Rhizobium etli		
USDA9043(pDT42)	2,046	542
USDA9043(pDTXC-12)	0	0
Rhizobium galegae		
USDA4128(pDT42)	2,338	51
USDA4128(pDTXC-12)	0	0
USDA4130(pDT42)	2,604	623
USDA4130(pDTXC-12)	0	0
Rhizobium huakuii		
USDA4773(pDT42)	2,763	537
USDA4773(pDTXC-12)	0	0
Rhizobium leguminosarum bv. phaseoli		
KIM5s(pT2TFXK)	2,457	478
KIM5s(pT2TX3K)	0	0
Rhizobium leguminosarum bv. trifolii		
T24	1,913	41
T24::Tn*5*$_1$	0	0
Rhizobium tropici		
USDA9030(pDT42)	1,746	59
USDA9030(pDTXC-12)	0	34
USDA9039(pDT42)	2,425	453
USDA9039(pDTXC-12)	0	0
Rhodospirillum rubrum UR2(pTFX3)	528	0

[a] Rifampin (50 μg/ml) and streptomycin were used to select against the *E. coli* donor in the *Brucella* and *Rhodospirillum* matings, respectively. In all other matings, the donor was selected against by using a minimal medium (7) in noble agar, which supports only poor growth of *E. coli* and good growth of the α-proteobacteria.

[b] For each strain number, the first and second transconjugant are TFX producing and non-TFX producing, respectively.

stored in Luria-Bertani broth and *Brucella* broth, respectively, each in 15% glycerol. The medium used to interrupt the matings was that used to culture the recipient (Table 2).

The TFX sensitivity assay used was as described previously (8) with minor modifications. The sources of TFX production were *R. leguminosarum* bv. trifolii T24 and *R. leguminosarum* bv. phaseoli KIM5s(pT2TFXK). The latter overproduces TFX compared with T24. This is presumably the result of multiple copies of pT2TFXK in the cell. As controls for TFX production in these experiments, we included T24::Tn*5*$_1$ (24) and KIM5s(pT2TX3K). Turbid suspensions (5 μl) at an optical density at 600 nm of 1 from the four strains were spotted in the center of agar plates containing the appropriate medium. After 2 days at 28°C, the plates were sprayed with a slightly turbid suspension (optical density at 600 nm of 0.1) of the strain to be tested for TFX sensitivity. No medium on which both *Brucella* and *Rhizobium* strains would grow well was found. Thus, TFX sensitivity of the *Brucella* strains was determined by using *Brucella* agar as an overlay on YM (27) plates which had been spotted with rhizobia 2 days earlier. After 2 days at 28°C, the area of the center colony was subtracted from the area of the inhibition zone to determine the net area of inhibition. In most assays, *R. leguminosarum* bv. viceae 128C1 was assayed along

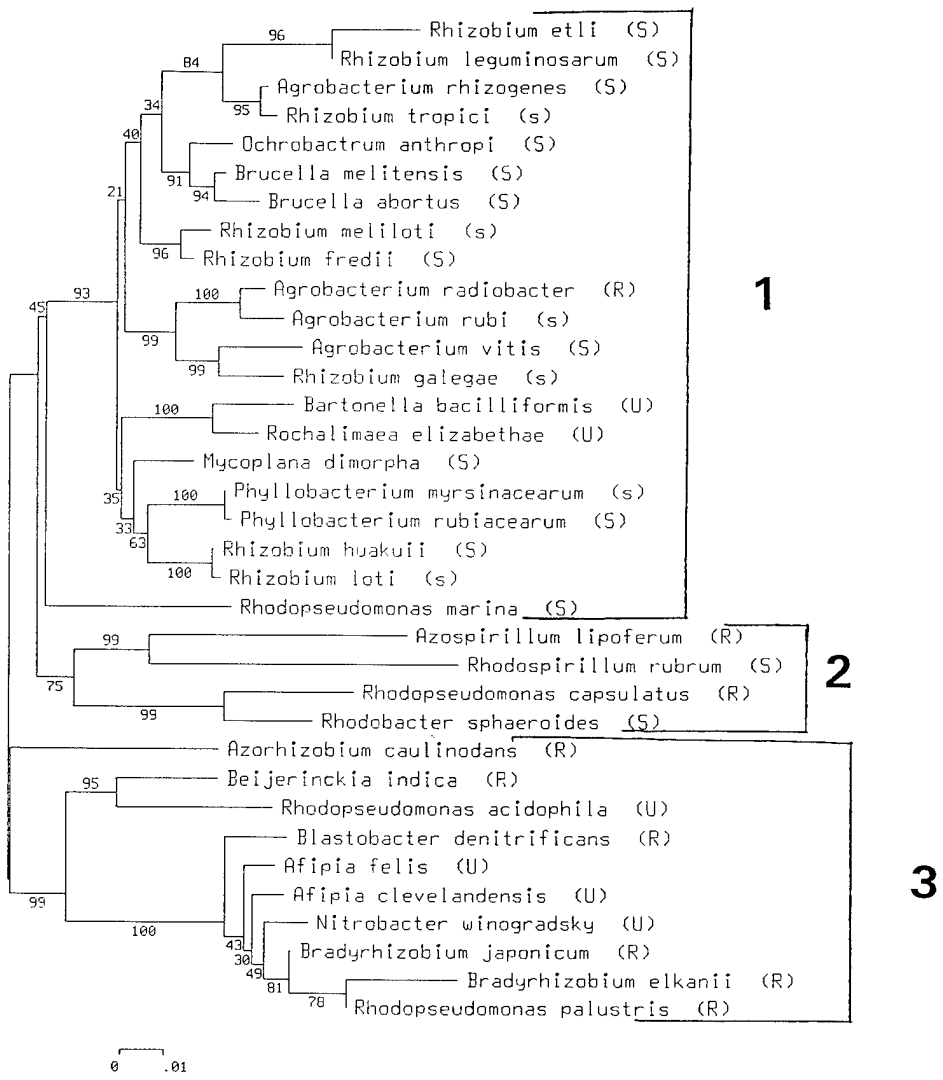

FIG. 1. Phylogenetic tree of full-length 16S rRNA sequences of selected α-proteobacteria prepared from sequences in the GenBank database by using the PILEUP program of the Genetics Computer Group package (3). The pairwise distance matrix and phylogenetic tree were constructed by using the Jukes-Cantor algorithm and the Neighbor-Joining method, respectively, in the MEGA package (13). Species names are flanked by a letter indicating sensitivity to TFX at levels produced by T24 (S) or an overproducing strain(s), resistance to TFX at either level of production (R), or unknown TFX sensitivity (U). Groups 1, 2, and 3 specify clusters of species which differ in TFX sensitivity. Group 1 and 3 species are nearly always TFX-sensitive and -resistant, respectively. Group 2 species are either sensitive or resistant to TFX. Confidence levels (percentages) shown above each node were generated from 500 bootstrap trees. The numbers of changes per sequence position are represented on the distance scale assuming a median rate of change. GenBank accession numbers for the 16S rRNA sequences are D12782, D12784, D12786, D12788, D12789, D12790, D12791, D12792, D12793, D12794, D12795, D12797, D12798, L01260, L11661, L11664, L20762, L26166, M27534, M32020, M34218, M34129, M55490, M59060, M59061, M65248, M65249, M69186, S46917, X13695, X53853, X66024, X67221, X67228, and X67228.

with other strains to provide an internal standard for our quantitative assay for TFX sensitivity.

The TFX sensitivities of several genera within the α-proteobacteria were determined (Table 2). Since our last survey of TFX inhibition of *Rhizobium* strains in 1987 (24), four new species of *Rhizobium* have been described. Strains of all known species of *Rhizobium* are inhibited by TFX at levels produced by either T24 or overproducing strains (Table 2). Several *Agrobacterium* strains were previously shown to be resistant to T24 levels of TFX (24). In this work, strains of *Agrobacterium rhizogenes* and *Agrobacterium vitis* were very sensitive to T24 levels of TFX and a strain of *Agrobacterium rubi* was sensitive to the overproducing strain (Table 2). Other α-proteobacterium species which are sensitive to T24 levels of TFX were found, including *Brucella abortus*, *Brucella melitensis*, *Mycoplana dimorpha*, *O. anthropi*, *Phyllobacterium rubiacearum*, *Rhodobacter sphaeroides*, *Rhodospirillum rubrum*, and *Rhodopseudomonas marina* (Tables 2 and 3). *Phyllobacterium myrsinacearum* is sensitive only to overproduction of TFX (Table 2). *Brucella abortus* RB51 was sensitive to T24 and KIM5s, with net inhibition zone areas of 537 and 844 mm^2, respectively. These data are not included in Table 2 because it was not possible to measure 128C1 and RB51 inhibition on the same medium.

Upon transfer of *tfx* to several *Rhizobium* strains by conjugation, TFX was produced by members of the four new species of *Rhizobium*: *Rhizobium etli*, *Rhizobium galegae*, *Rhizobium huakuii*, and *Rhizobium tropici* (Table 3). TFX production by *Brucella abortus*, *A. rhizogenes*, *P. rubiacearum*, and *Rhodospi-*

rillum rubrum following transfer of either pDT42 or pTFX3 was also observed (Table 3).

The phylogeny of TFX sensitivity correlates well with the phylogeny of the α-proteobacteria on the basis of 16S rRNA sequences (Fig. 1). There are three groups within the α-proteobacteria which differ in TFX sensitivity (Fig. 1). Group 1 species, with rare exceptions, are sensitive to TFX. Group 2 includes species that differ widely in TFX sensitivity. Group 3 includes only strains which are resistant to TFX. Strains within this third group are often resistant to many antibiotics (9, 15). Thus, the taxonomic limits of TFX sensitivity are confined to two groups within α-proteobacteria which are closely related genetically but inhabit very different environments. This allows us to predict whether a species within the α-proteobacteria is sensitive to TFX. For example, we predict that group 1 bacteria such as the animal pathogens *Rochalimaea* and *Bartonella* spp. are inhibited by TFX while group 3 genera such as *Afipia* and *Nitrobacter* are resistant to TFX.

An understanding of the taxonomic relationships of antibiotic-producing organisms can lead to entirely new applications for antibiotics. A potential agricultural application of the TFX system as a means to limit nodulation of legume roots by indigenous, TFX-sensitive rhizobia has been described (21–24). The ability of TFX to inhibit *Agrobacterium*, *Brucella*, and *Ochrobactrum* spp. may lead to new treatments for infections with these animal and plant pathogens. The ability of *Brucella abortus* RB51(pDT42) to produce TFX shows that genes from *Rhizobium* spp. can be expressed in *Brucella* spp., permitting extensive genetic comparisons between these two genera. Also, the use of TFX-producing strains under agricultural conditions may have broad ecological implications because a far wider spectrum of bacteria are inhibited than previously thought.

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$CD8^+$ Type 1 $CD44^{hi}$ $CD45\ RB^{lo}$ T lymphocytes control intracellular *Brucella abortus* infection as demonstrated in major histocompatibility complex class I- and class II-deficient mice

Genetically engineered mice with a targeted disruption in the β2-microglobulin (β2-m) gene or the H2-I-Aβ chain (Aβ) which lack functional $CD8^+$ or $CD4^+$ T cells, respectively, were used to assess the role of T cell subsets in *Brucella abortus* infection. Murine brucellosis was markedly exacerbated in β2-m-deficient mice ($\beta2\text{-}m^{-/-}$) compared to Aβ mutant ($A\beta^{-/-}$) or C57BL/6 mice, strongly indicating that optimal resistance to *B. abortus* requires $CD8^+$ T cells. Splenocytes from *Brucella*-primed $\beta2\text{-}m^{-/-}$, $A\beta^{-/-}$ and C57BL/6 mice exhibited a type 1 cytokine profile marked by elevated IFN-γ mRNA expression and protein production, and basal levels of IL-2 and IL-4 transcripts. *B. abortus* did not induce secretion of TGF-β1, but substantial IL-10 activity was detected in spleen cell supernatants from all mouse strains studied. $CD8^+$ T cells from $A\beta^{-/-}$ and C57BL/6 mice displayed a $CD44^{hi}$ $CD45RB^{lo}$ phenotype and a type 1 cytokine transcription profile featuring high levels of IFN-γ mRNA. Additionally, we have shown the ability of C57BL/6 $CD8^+$ CTL to kill *Brucella*-infected macrophages. This study illustrates the predominant role of MHC class I-restricted T cells in controlling *B. abortus* infection.

1 Introduction

Brucella abortus is a facultative intracellular bacterium that infects human and domestic animals [1]. The pathological manifestations of brucellosis are diverse and include arthritis, endocarditis and meningitis in humans, while bovine brucellosis is characterized by spontaneous abortion [2]. Previously, immunity to intracellular bacteria was considered to be exclusively mediated by $CD4^+$ T cells [3]. Recently, optimal protection to intracellular bacteria is regarded as a coordinated interaction between different T cell subsets [4]. Additional studies have evoked a central role for $CD8^+$ T lymphocytes in conferring protective immunity against intracellular pathogens [5–9]. However, the principal mechanism by which $CD8^+$ T cells coordinate anti-microbial resistance remains to be determined. Particularly, in murine brucellosis, earlier studies focusing on the importance of T cell subsets in the resolution of infection generated conflicting data suggesting either CD4 [10], CD8 [10–12], or both were responsible for protection. The paradigm of disease resolution was founded on experiments using adoptive transfer of immune T cells or depletion of these subpopulations using mAb *in vivo*. Technically, such experimental approaches have potential pitfalls. Antibody treatment may leave a small but active population of T cells unaffected, while adoptively transferred immune T cells may not efficiently home to the site of infection. Alternatively, transferred cells may interact with residual T cells in the recipient animal by cytokine production, confounding the identification of the immune cells required for protection [13].

To clarify the controversy surrounding the nature of the T cell subsets responsible for *B. abortus* disease resolution, we used gene knockout mice. Mice in which the β2-microglobulin (β2-m) gene was disrupted or the MHC class II Aβ chain was deleted were recently constructed [14, 15]. These mutations result in a failure to assemble and express either MHC class I or class II molecules on the cell surface. The lack of MHC class I and class II molecules impairs positive thymic selection, resulting in animals devoid of functional $CD8^+$ or $CD4^+$ αβ T cells, respectively. Therefore, these gene knockout mutants provide a useful approach for studying the role of T cell subsets in microbial immunity.

Thus, this study using β2-m and Aβ deficient mice was designed to elucidate the contribution of $CD4^+$ or $CD8^+$ T lymphocytes in controlling *B. abortus* infection. In addition, the cytokine transcription profile as well as secreted cytokines were assayed to define the role of these molecules in immunoregulation of bacterial replication. Finally, we tested the ability of $CD8^+$ CTL from Aβ mutant or C57BL/6 mice to kill *B. abortus*-infected macrophages as a mechanism of protective immunity. The results reached in this study illustrate the pivotal role played by $CD8^+$ T cells in resolving *B. abortus* infection. Moreover, our findings provide insight into mechanisms used by $CD8^+$ T cells to control an intracellular bacterial infection which are of major interest for vaccine development.

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Abbreviations: **$\beta2\text{-}m^{-/-}$:** β2-microglobulin deficient **$A\beta^{-/-}$:** MHC class II deficient **p.i.:** Post-infection **RT-PCR:** Reverse transcriptase-PCR

Key words: T cell subsets / $CD8^+$ T cells / Infection / *Brucella abortus* infection / Cytokine regulation / Major histocompatibility complex class I and class II deficient mice

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2 Material and methods

2.1 Mice

Mice homozygous for the β2-microglobulin mutation (β2-$m^{-/-}$) were kindly provided by E. Balish and D. Muller (University of Wisconsin, Madison, WI) and later purchased from The Jackson Laboratory (Bar Harbor, ME). The MHC class II-deficient mutants ($A\beta^{-/-}$) were kindly provided by W. P. Weidanz (University of Wisconsin, Madison, WI) and female C57BL/6 were purchased from The Jackson Laboratory and used as controls. All animals were housed in a Biosafety Level (BL)-3 facility and handled according to University of Wisconsin-Madison Research Animal Resource Center guidelines. Male and female mutant mice were used between 8–14 weeks of age. In all experiments, homozygous mutants were backcrossed to C57BL/6 mice, and F1 mice were used.

2.2 Bacteria and mouse infection

Brucella abortus, live attenuated vaccine strain 19, was obtained from B. Martin (National Animal Disease Center, Ames, IA) at 2×10^{10} colony-forming units (CFU)/ml. Mice received an i.v. injection of 1×10^6 CFU *B. abortus* in 100 μl sterile PBS. For use as Ag in T cell cultures to measure cytokine production, the bacteria (5×10^8 CFU/ml) were killed by γ-irradiation with ^{137}Cs (250 000 rad).

2.3 Quantitation of bacteria in the spleen

Mice were killed by cervical dislocation. Spleens were homogenized in plastic bags in sterile PBS using a Stomacher Lab Blender (Tekmar, Cincinnati, OH), serially diluted ten-fold and plated on *Brucella* agar (Difco, Detroit, MI). Colonies were counted after incubation for 3 days at 37 °C under 5 % CO_2 in air.

2.4 Cell depletion *in vivo*

Groups of mice were depleted of $CD4^+$ or $CD8^+$ T cells by injection of 1 mg mAb anti-L3T4 (GK 1.5) or anti-Lyt-2 (2.43) (American Type Culture Collection, Rockville, MD) i.p. on days −2, 1, 4, 7, 10 after infection. Briefly, antibodies were prepared as ascites in pristane-primed nude mice, purified using a Microtainer serum separator (Becton Dickinson, Rutherford, NJ) and a lipid-clearing solution (Clinetics, Tustin, CA), and sterilized by filtration through a 0.22 μm membrane (Millipore, Bedford, MA). Efficacy of cell depletions determined by flow cytometric analysis was greater than 99 %.

2.5 Flow cytometric analysis and cell sorting

Single-cell suspensions were prepared from the spleen of individual infected and naive mice and prepared for flow cytometric analysis as described [16]. Briefly, samples containing 1×10^6 cells were incubated for 30 min on ice with a specific mAb. After extensive washing the cells were resuspended in PBS-BSA 1 % and further analyzed by flow cytometry. Monoclonal antibodies of similar isotypes but specific for unrelated Ag were used as negative controls. The following directly conjugated anti-mouse antibodies were used: PE-labeled anti-L3T4 (GK 1.5), FITC-labeled anti-Lyt-2 (53-6.7) (Becton Dickinson, San Jose, CA) and PE-anti-γδ TcR (GL3), anti-αβ TcR (H57-597), anti-NK1.1 (PK136), anti-B220/CD45 (RA3-6B2), anti-CD44 (IM7), and anti-CD45RB (23G2) (PharMingen, San Diego, CA).

For cell sorting of $CD8^+$ T cells, 1×10^8 splenocytes were labeled with anti-Lyt-2 (53-6.7) and sorted using a FACScan Plus cell sorter (Becton Dickinson, Mountain View, CA). The purity of the $CD8^+$ T cells sorted was typically ≥ 97 %.

2.6 Competitive quantitative reverse transcriptase (RT)-PCR

Total RNA was extracted from splenocytes or $CD8^+$ T cells using guanidinum thiocyanate (TRI reagent, Molecular Research Center, Cincinnati, OH) according to the manufacturer's directions. RNA (2 μg) were subjected to first-strand cDNA synthesis in a 30-μl reaction as described [16]. After reverse transcription, the specific cDNA template was diluted from 30 to 100 μl, and 2 μl of this preparation was used as template in a 50-μl PCR reaction. Specific primers and competitors for IL-2, IL-4, IFN-γ and β-actin were purchased from Clontech (Palo Alto, CA), and the PCR reaction was performed according to manufacturer's directions. To detect the amplified products, 6 μl of the final reaction mix was run on a 1 % agarose gel with molecular weight standards at 100 V for 1 h. The ethidium bromide (0.5 mg/ml)-prestained gel was visualized under UV light and photographed. To quantify the amount of cytokine mRNA expressed compared to competitive internal standards, we used a video system (NIH image 1.54) to scan and quantitate the ethidium bromide-stained gel bands.

2.7 ELISA

Cytokines were detected in the supernatant of splenocytes using an ELISA specific for mouse IL-2, IL-4, IFN-γ, IL-10 (Endogen, Boston, MA) and human TGF-β1, which is cross-reactive with murine TGF-β1 (Genzyme, Cambridge, MA). The assays were performed in duplicate according to the manufacturer's directions.

2.8 ^{51}Cr-release assay

The activity of $CD8^+$ CTL was determined by a ^{51}Cr-release assay. Splenic macrophages from $A\beta^{-/-}$ and C57BL/6 mice were pre-activated with 100 U/ml of murine rIFN-γ followed by overnight incubation with 1×10^8/ml live *B. abortus* at 37 °C in 5 % CO_2 in air. Infected macrophages were washed twice with RPMI 1640 (Sigma, St. Louis, MO) containing 70 μg/ml streptomycin and 120 μg/ml gentamycin. $CD8^+$ sorted T cells (5×10^6) from infected $A\beta^{-/-}$ or C57BL/6 mice were incubated with syngeneic infected macrophages for 6 days in medium containing 100 U/ml IL-2 (Genzyme) to stimulate and expand *B. abortus*-

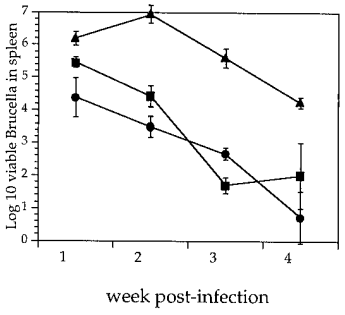

Figure 1. Course of *B. abortus* infection in β2-m$^{-/-}$, Aβ$^{-/-}$ and C57BL/6 mice. The graph illustrates *B. abortus* CFU in the spleen determined at different times after infection. Data are expressed as means ± SE of four animals per time point. C57BL/6 (■), Aβ$^{-/-}$ (●), and β2-m$^{-/-}$ (▲).

specific CTL. After culture, CD8$^+$ T cells were harvested and used as effector cells. As a source of target cells, splenic macrophages (2×10^6) were infected as described above and labeled with 250 μCi sodium [^{51}Cr]chromate (DuPont, Boston, MA) for 90 min at 37 °C. After washing, 1×10^4 target cells/well were cultured in 96-well plates with effector CTL at the indicated E/T ratios in a final volume of 200 μl for 4 h at 37 °C. Spontaneous release was determined in wells containing target cells but without CTL. Maximum release was determined by the addition of 10 % SDS to wells containing only target cells. After 4 h, plates were centrifuged and 100 μl ^{51}Cr-containing supernatant was measured by gamma spectrophotometry. The percent specific lysis was calculated as follows: (experimental release − spontaneous release)/(maximal release − spontaneous release) × 100.

3 Results

3.1 *B. abortus* infection in MHC class I- and class II-deficient mice

To determine the contribution of CD4$^+$ or CD8$^+$ T cells in controlling murine brucellosis, bacterial numbers were monitored in the spleen of β2-m$^{-/-}$, Aβ$^{-/-}$, and C57BL/6 mice for 4 weeks after *B. abortus* infection (Fig. 1). The animals were killed weekly, and numbers of *B. abortus* CFU were determined. Interestingly, murine brucellosis was markedly exacerbated in animals that lacked functional CD8$^+$ T cells (β2-m$^{-/-}$) compared to Aβ$^{-/-}$ or C57BL/6 mice. After the first week post-infection (p.i.), *B. abortus* CFU increased in the spleens of β2-m$^{-/-}$ mice, whereas Aβ$^{-/-}$ mice displayed a constant decline in bacterial numbers throughout the course of infection. Additionally, the number of *B. abortus* CFU in β2-m$^{-/-}$ mouse spleens were at least two logs higher than those observed in spleens of class II-deficient mice during the 4 weeks p.i. Fig. 1 clearly demonstrates the impact of CD8$^+$ T cells in cell mediated immunity in murine brucellosis. To reinforce the importance of CD8$^+$ T cells in resistance to infection, depletion *in vivo* of CD8$^+$ T cells from Aβ$^{-/-}$ and C57BL/6 mice, as well as depletion of CD4$^+$ T cells from β2-m$^{-/-}$ and C57BL/6 mice, were performed. Depletion of CD8$^+$ T cells caused a dramatic increase in bacterial numbers in the spleens of Aβ$^{-/-}$ and C57BL/6 mice, as shown in Fig. 2. In contrast, anti-L3T4 mAb treatment in β2-m$^{-/-}$ and C57BL/6 mice actually decreased *B. abortus* CFU in the spleen of both mouse strains. Further, Fig. 2 confirms the observations in Fig. 1 that β2-m$^{-/-}$ mice not treated with mAb displayed a marked susceptibility to brucellosis compared to Aβ$^{-/-}$ and C57BL/6 mice, suggesting the necessity of CD8$^+$ T cells for optimal resistance to *B. abortus* infection.

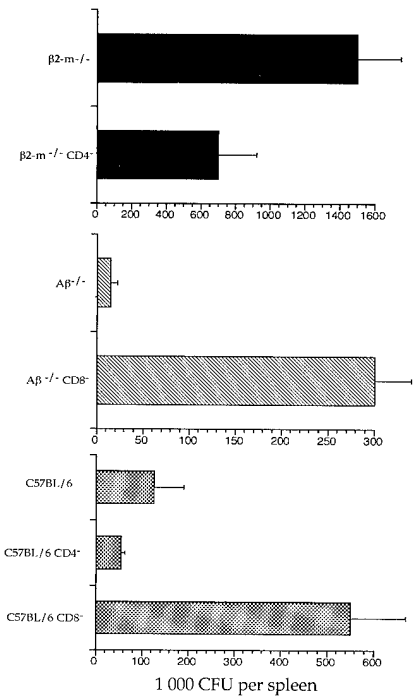

Figure 2. Depletion *in vivo* of CD4$^+$ T cells (CD4$^-$) in β2-m$^{-/-}$ or C57BL/6 mice and CD8$^+$ T cell depletion (CD8$^-$) in Aβ$^{-/-}$ or C57BL/6 mice. Mice were injected i.p. with specific mAb at different time points and killed 15 days later to determine bacterial numbers per spleen. The data are expressed as means ± SE of four animals per group.

3.2 Spleen cell populations during *B. abortus* infection

To determine changes in cell populations during the course of murine brucellosis, flow cytometric analysis was performed in splenocytes from all three mouse strains. As expected, microfluorimetric analysis revealed low numbers of CD4$^+$ (≤ 3.5 %) and CD8$^+$ (≤ 2 %) T lymphocytes in spleens of Aβ$^{-/-}$ and β2-m$^{-/-}$ mice, respectively (Fig. 3). In addition, we have confirmed a striking increase (at least 50 %) in the percentage of CD8$^+$ T cells in spleens of Aβ$^{-/-}$ mice compared to C57BL/6 mice [15]. No significant change in the numbers of B220$^+$ cells in the spleens of all three mouse strains was detected. However, a three-fold increase in γδTcR and NK1.1$^+$ cell percentages was observed after the second week post-infection in β2-m$^{-/-}$ splenocytes, suggesting a possible role for these cell types in the increased resistance to *B. abortus* during this period, as demonstrated in Fig. 1. No dramatic change was detected in the numbers of γδTcR and NK1.1$^+$ splenocytes in Aβ$^{-/-}$ and C57BL/6 mice.

3.3 Cytokine analysis

Cytokines are key molecules that play a major role in shaping the development of protective or noncurative immune responses [17]. To determine which of these important regulatory molecules are involved in orchestrating the cellular immune response in brucellosis, the cytokine transcription profile as well as secreted products were assayed. Splenocytes from infected and naive animals were harvested and the cytokine mRNA pattern was determined by a competitive quantitative PCR assay. The results of the

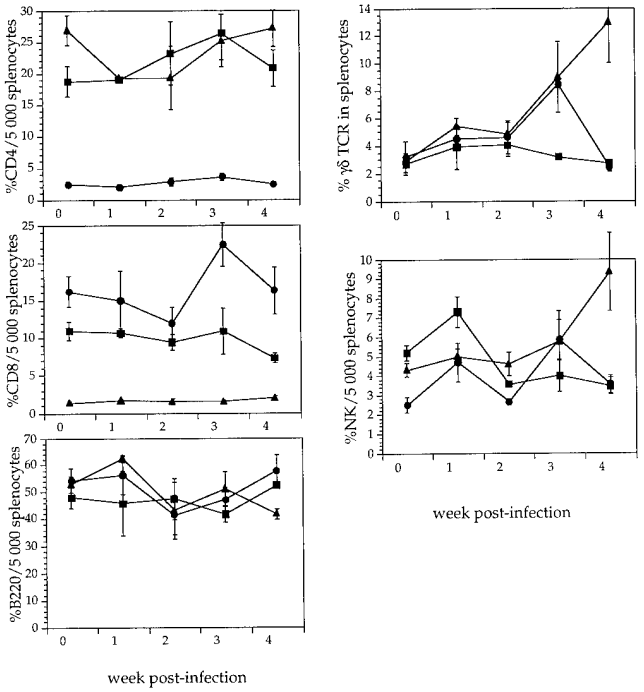

Figure 3. Changes in spleen cell populations of β2-m$^{-/-}$, Aβ$^{-/-}$ and C57BL/6 mice during *B. abortus* infection. Cell numbers were determined by flow cytometric analysis on groups of four animals per time point. The data are expressed as means ± SE. C57BL/6 (■), Aβ$^{-/-}$ (●), and β2-m$^{-/-}$ (▲).

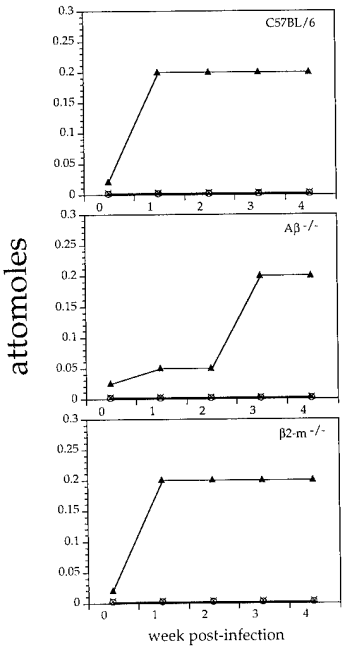

Figure 4. Kinetics of IL-2, IL-4 and IFN-γ gene expression in spleen cells from β2-m$^{-/-}$, Aβ$^{-/-}$ or C57BL/6 mice during the course of *B. abortus* infection. Data are expressed as means of three animals per time point. Standard errors are omitted, and did not exceed 10% of the mean values. Quantitation of β-actin mRNA was measured in all experiments as a positive control (data not shown). IL-2 (×), IL-4 (○), and IFN-γ (▲).

mRNA analyses for a panel of cytokines demonstrated that *Brucella*-primed splenocytes from all three mouse strains exhibited by type 1 cytokine profile (Fig. 4). Up-regulation of mRNA transcripts for IFN-γ, but not IL-2 or IL-4, were detected. C57BL/6 and β2-m$^{-/-}$ mouse splenocytes displayed a ten-fold increase in the level of IFN-γ mRNA expressed compared to splenocytes from naive animals. Interestingly, Aβ$^{-/-}$ mouse splenocytes showed only a two-fold elevation of IFN-γ transcripts within the first 2 weeks after *B. abortus* infection. However, IFN-γ transcription in Aβ$^{-/-}$ mouse splenocytes reached levels similar to those of the other strains after 2 weeks p.i. To confirm that the transcriptionally active cytokines were also secreted from Ag-activated spleen cells, ELISA for IL-2, IL-4 and IFN-γ were performed. As expected, IL-2 and IL-4 (data not shown) production were not detected in culture supernatants when splenocytes from all three mouse strains were cultured with γ-irradiated *B. abortus* or medium alone (Fig. 5A). However, IFN-γ was secreted from spleen cells from all mouse strains when the cells were cultured with γ-irradiated *B. abortus*. Aβ$^{-/-}$ mouse splenocytes produced less IFN-γ than spleen cells from β2-m$^{-/-}$ or C57BL/6 mice (Fig. 5B). These results established a strong correlation between the level of cytokine mRNA transcripts expressed in spleen cells and the pattern of protein secreted in the splenocyte culture supernatants. To elucidate the cytokine network involved in murine brucellosis and its possible role in enhancing resistance or susceptibility to *B. abortus*, we measured IL-10 and TGF-β1 in splenocyte culture supernatants by ELISA. IL-10 and TGF-β1 are cytokines known to be involved in down-regulating protective immune responses by inhibiting Th1 cell differentiation or by blocking cytokine-induced macrophage activation [18, 19]. Interestingly, *B. abortus* infection did not induce production of TGF-β1 by splenocytes from any of the three mouse strains studied: levels of TGF-β1 secreted by Ag-activated spleen cells were similar to the amount produced by cells cultured with medium alone (data not shown). In contrast, substantial amounts of IL-10 were detected in splenic culture supernatants at 1 week p.i. from all three mouse strains. Surprisingly, spleen cells from β2-m$^{-/-}$ mice lacking functional CD8$^+$ T cells produced two-fold higher levels of IL-10 compared to splenocytes from Aβ$^{-/-}$ or C57BL/6 mice (Fig. 5C).

3.4 CD44 and CD45 RB membrane expression on CD8$^+$ T cells

The importance of CD8$^+$ T cells in controlling *B. abortus* infection is emphasized by the data of Figs. 1 and 2. We next examined changes in expression of the T cell activation and homing markers CD44 and CD45RB on CD8$^+$ T cells from Aβ$^{-/-}$ and C57BL/6 mice by flow cytometric analysis. Kinetics of CD44 and CD54RB surface expression on CD8$^+$ T cells at 0, 1 and 2 weeks p.i. is shown in Table 1. CD8$^+$ T lymphocytes from Aβ$^{-/-}$ and C57BL/6 mice had a similar time-dependent shift in CD44 and CD45RB expression. Up-regulation of CD44 expression (CD44^{hi}) and down-regulation of CD45RB expression (CD45RB^{lo}) was observed when naive cells were activated by *B. abortus*. The time-dependent change in phenotype from CD8$^+$ CD44^{lo} CD45RB^{hi} to CD8$^+$ CD44^{hi} CD45RB^{lo} is a feature of cell activation within this population [20] and consistent with the critical role played by CD8$^+$ T cells in controlling murine brucellosis. Additionally, flow cytometric analysis revealed that ≥ 95% of sorted CD8$^+$ T cells possess the αβ TcR, and we have correlated the CD44^{hi} CD45RB^{lo} phenotype with the ability of CD8$^+$ T cells to express IFN-γ transcripts, as described below.

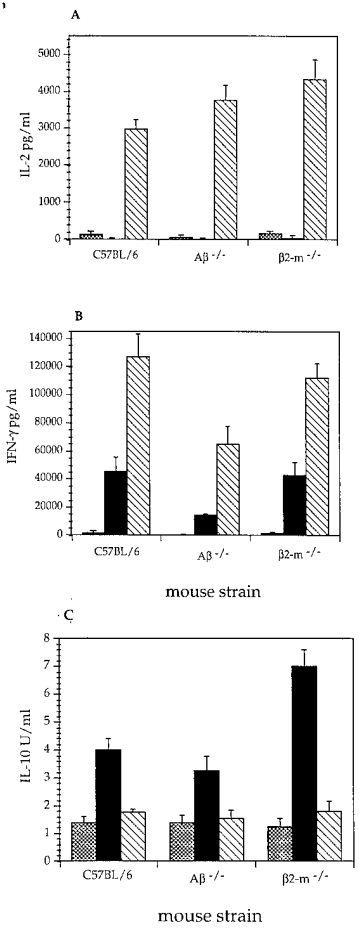

Figure 5. Cytokine responses of primed splenocytes from β2-m$^{-/-}$, Aβ$^{-/-}$ or C57BL/6 mice *in vitro* restimulated with γ-irradiated *B. abortus* or Con A (2.5 μg/ml) for 48 h. Mice were infected with 1×10^6 CFU and sacrificed 1 week post-infection. Single-cell suspensions were prepared from spleens of infected animals and cultured with the indicated Ag. Levels of IL-2 (A), IFN-γ (B), and IL-10 (C) present in cell culture supernatants were assayed by ELISA. Data are expressed as means ± SE of two animals. Treatment groups included medium (shaded bars), γ-irradiated *B. abortus* (solid bars), and Con A (hatched bars).

3.5 Cytokine gene expression in *B. abortus*-primed CD8$^+$ T cells

To define better the mechanisms by which CD8$^+$ T cells control *B. abortus* infection, a cytokine transcription profile of this T cell subpopulation was determined. CD8$^+$ T lymphocytes from Aβ$^{-/-}$ or C57BL/6 splenocytes were sorted at 0 and 1 week after *B. abortus* infection using fluorescence-activated cell sorting. Total RNA was extracted from CD8$^+$ T cells immediately following sorting. Competitive RT-PCR was performed to assess expression levels of IL-2, IL-4, IFN-γ and β-actin mRNA. CD8$^+$ T cells from *B. abortus*-infected Aβ$^{-/-}$ or C57BL/6 mice demonstrated no increase in mRNA expression for IL-2 or IL-4 compared to CD8$^+$ T cells from untreated mice (data not shown). However, IFN-γ mRNA was markedly elevated in CD8$^+$ T cells from both *B. abortus*-infected mouse strains when compared with naive groups. In addition, *B. abortus*-primed CD8$^+$ T cells from Aβ$^{-/-}$ mice expressed ten-fold higher levels of IFN-γ transcripts than C57BL/6 CD8$^+$ T lymphocytes. Thus, bacterially primed CD8$^+$ T cells from Aβ$^{-/-}$ or C57BL/6 mice exhibited a type 1 cytokine profile identical to the pattern displayed by unseparated spleen cells from infected animals.

3.6 Cytotoxic activity of *B. abortus*-induced CD8$^+$ T cells

The ability of *B. abortus*-specific CD8$^+$ T cells from Aβ$^{-/-}$ or C57BL/6 mice to lyse infected Aβ$^{-/-}$ or C57BL/6 splenic macrophages, respectively, was assayed. Specific lysis of

Table 1. Kinetics of CD44 and CD45RB membrane expression on CD8$^+$ T cells during *B. abortus* infection

Group of mice[a)]	% staining positive[b)]	
	CD44	CD45RB
C57BL/6		
Naive	18.6 ± 2.4	90.9 ± 1.4
1 week post-infection	43.2 ± 4.7	24.5 ± 1.7
2 weeks post-infection	54.6 ± 2.2	16.5 ± 3.3
Aβ$^{-/-}$		
Naive	11.3 ± 0.9	87.8 ± 4.2
1 week post-infection	38.9 ± 2.9	34.5 ± 1.6
2 weeks post-infection	91.2 ± 7.2	13.6 ± 1.1

a) Three animals were in each group.
b) Splenic CD8$^+$ T cells from individual mice in each group were analyzed by flow cytometry for marker expression. Data are expressed as means ± SE.

infected C57BL/6 macrophages was detected in all three different E/T ratios used (Fig. 6) Minimal lysis was observed when CD8$^+$ T cells were incubated with uninfected C57BL/6 macrophages. Contrary to our expectation, Aβ$^{-/-}$ CD8$^+$ T cells were unable to lyse infected Aβ$^{-/-}$ macrophages in the same fashion (Fig. 6).

4 Discussion

This study demonstrates the predominant role of CD8$^+$ T cells in controlling experimental brucellosis in mice. β2-m$^{-/-}$ mice demonstrated an enhanced susceptibility to *B. abortus* infection, while Aβ$^{-/-}$ mice that possess CD8$^+$ T cells controlled infection as efficiently as C57BL/6 mice. Additionally, depletion of CD8$^+$ T cells *in vivo* in Aβ$^{-/-}$ and C57BL/6 mice abrogated resistance to brucellosis, whereas anti-L3T4 mAb treatment in β2-m$^{-/-}$ and C57BL/6 mice surprisingly decreased *B. abortus* CFU in mouse spleens. Also, our data suggest that a combination of mechanisms underlie the acquisition of protective immunity against this intracellular bacterium by CD8$^+$ T cells. First, Aβ$^{-/-}$ and C57BL/6 CD8$^+$ T cells exhibited a type 1 cytokine profile, marked by production of IFN-γ, a critical cytokine involved in host protection. Second, mice lacking CD8$^+$ T cells (β2-m$^{-/-}$) produced two-fold higher levels of IL-10, an immuno-

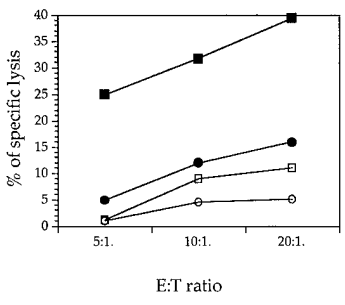

Figure 6. Lysis of *B. abortus*-infected macrophages by CTL in a ^{51}Cr-release assay. Two weeks post-infection, CD8$^+$ T cells from Aβ$^{-/-}$ or C57BL/6 mice were sorted and CTL were generated by restimulation with specific Ag *in vitro*. Target cells were syngeneic *B. abortus*-infected or uninfected splenic macrophages. Treatment groups included C57BL/6 + Ag (■), C57BL/6 (□), Aβ$^{-/-}$ + Ag (●), and Aβ$^{-/-}$ (○).

suppressive cytokine [21] compared to the other mouse strains evaluated. Third, primed $CD8^+$ T cells were able to lyse *Brucella*-infected macrophages.

Previously, immunity to intracellular bacteria was considered to be exclusively dependent on MHC class II-restricted $CD4^+$ T cells [3]. However, recent findings have emphasized a major role of MHC class I-restricted $CD8^+$ T cells in protection against intracellular bacteria [8, 22, 23]. In murine brucellosis, an early study demonstrated that $CD4^+$ and $CD8^+$ T cells provide an equivalent level of protection [10]. Others have questioned these results, suggesting that *Brucella*-primed $CD4^+$ T cells mediate inflammatory reactions like DTH, splenomegaly and granulomatous inflammation, whereas $CD8^+$ T cells mediate protection [12]. These previous studies were performed using mAb to deplete T cell subsets and adoptive transfer of immune T cells. Both technical approaches are inherently limited. However, genetically constructed mouse strains that lack the ability to express functional MHC class I and class II molecules provide powerful tools to define the role of T cell subsets in microbial immunity.

T lymphocytes recognize antigenic peptides in association with MHC molecules. $CD4^+$ T cells recognize endosome-derived peptides in association with MHC class II molecules, whereas $CD8^+$ T cells are biased towards cytosolic peptides seen in the context of MHC class I molecules [23]. Therefore, translocation of *Brucella* antigens from the endosome into the cytosol is required for the stimulation of *Brucella*-specific MHC class I restricted $CD8^+$ T cells. Interestingly, *B. abortus* strain 2308 inhibits phagosome-lysosome fusion and can be found in the endoplasmic reticulum of infected nonphagocytic cells, which would be an optimal localization for presentation through the MHC class I pathway [24]. However, to our knowledge, the specific mechanism by which *Brucella* Ag are processed and presented to MHC class I restricted $CD8^+$ T cells has yet to be established.

Our results reveal the pivotal role of $CD8^+$ T cells in controlling *B. abortus* infection. In *Mycobacterium tuberculosis* or *Listeria monocytogenes* infection similar conclusions have been reached when $\beta2\text{-m}^{-/-}$ mice were used [25, 26]. Although β2-m deficiency had much more dramatic consequences on *B. abortus* replication than the lack of MHC class II, $CD4^+$ T cells are likely to play a helpful, but not essential role against *Brucella* infection. Since an enhanced resistance was observed in $\beta2\text{-m}^{-/-}$ 2 weeks p.i., we postulate a modest participation of MHC class II-dependent T cells in protection. Further, we cannot rule out the possibility of additional roles played by expanded γδTcR and $NK1.1^+$ populations in $\beta2\text{-m}^{-/-}$ mice, as observed in Fig. 3. Ladel et al. [4] employed for the first time MHC class II-deficient mice to analyze immunity against bacterial infection, concluding that optimal protection to *L. monocytogenes* depends on a coordinated interplay between different T cell subsets. Our findings diverge to some extent from Ladel's results, and we further examined the role of $CD8^+$ T cells in intracellular bacterial infection. Our data, therefore, clearly show that $CD8^+$ T cells are required for optimal protection against *B. abortus* infection. Having gained a better understanding of the predominant role performed by MHC I-restricted T cells in brucellosis, we investigated the possible functions of $CD8^+$ T cells in protection. Classically, $CD8^+$ T cells are regarded as cytotoxic T lymphocytes. Recently, however, several lines of evidence suggest an additional immunoregulatory role for this T cell subset [27].

IFN-γ is a key cytokine that plays a prominent function in up-regulation of macrophage anti-*Brucella* activity and is considered crucial for protection against *B. abortus* [28, 29]. IFN-γ also promotes the development of Th1 cells [30]. In our experiments, *B. abortus* infection induces a type 1 cytokine transcription profile in $A\beta^{-/-}$ and C57BL/6 $CD8^+$ T cells, with high levels of IFN-γ mRNA and no IL-2 or IL-4. This profile parallels the pattern of cytokines expressed and secreted from *Brucella*-primed splenocytes, suggesting that Ag-induced IFN-γ production by $CD8^+$ T cells is involved in immunity against *B. abortus*. Despite the fact that $\beta2\text{-m}^{-/-}$ splenocytes secreted high levels of IFN-γ, this mouse strain was more susceptible to *B. abortus* infection than $A\beta^{-/-}$ or C57BL/6. This result confirms the assumption that in addition to IFN-γ production, immunity to intracellular bacteria usually requires the combination of different elements of the immune system to orchestrate the cellular response leading to protection [22]. Additionally, our observation that $CD8^+$ T cells are activated early in the immune response places them in an ideal position to regulate $CD4^+$ T cell development by producing IFN-γ or other regulatory cytokines (data not shown). Moreover, we have also confirmed recent findings that preactivated/memory $CD8^+$ T cells possessing a $CD44^{hi}$ $CD45RB^{lo}$ phenotype produce increased levels of IFN-γ compared to naive cells [31, 32].

IL-10 is another important cytokine known to down-regulate protective immunity to *B. abortus* by affecting macrophage effector functions and IFN-γ production [21]. The immune suppressor effect of IL-10 in infected hosts is also demonstrated in other infectious processes [33, 34]. In our system, *Brucella*-infected $\beta2\text{-m}^{-/-}$ spleen cells produced twice as much IL-10 as $A\beta^{-/-}$ or C57BL/6 splenocytes. In addition, $\beta2\text{-m}^{-/-}$ mice demonstrated an enhanced susceptibility to murine brucellosis compared to the other strains. The findings suggest a possible effect of IL-10 production in $\beta2\text{-m}^{-/-}$ mice, exacerbating *B. abortus* infection. It is also interesting that the augmentation in IL-10 production occurred in an animal lacking $CD8^+$ T lymphocytes. A recent study suggested a role for $CD8^+$ T cells in modulating the development of Th2 cells by down-regulating IL-4, IL-5 and IL-10 expression [35]. More specifically, in *L. monocytogenes* infection, Flesch and Kaufmann [36] demonstrated that αβ T lymphocytes inhibited macrophage IL-10 production. According to our data, we hypothesize that $CD8^+$ T cells may inhibit IL-10 production, and, as a consequence, murine brucellosis was markedly exacerbated in $\beta2\text{-m}^{-/-}$ mice. However, new experiments are required to prove this observation. MHC I-restricted T cells modulating type 2 cytokines like IL-10, known to down-regulate a protective immune response, might be another mechanism by which $CD8^+$ T cells control murine brucellosis. Additionally, our data corroborates the postulate that early high IL-10 levels produced in murine infectious models dominated by Th1 responses are not sufficient to direct Th0 differentiation to the Th2 pathway [36].

Pathogenesis induced by intracellular organisms is the product of a complex series of interactions between the pathogen, the infected host cell, and different elements of the

immune system [37]. One interaction of interest is between $CD8^+$ CTL and *Brucella*-infected macrophages. The role of $CD8^+$ CTL in immunity to *Brucella* has been the subject of less study. The major focus of protective immunity against *B. abortus* has dealt with IFN-γ production by Th1 cells [16, 38]. In the *Mycobacterium* model, Kaufmann suggested a possible mechanism by which cytotoxic T cells can enhance protective immunity [39]. First, both the pathogen and host cell could be killed by effector CTL. Second, the host cell could be lysed and the pathogen released, to be ingested and killed by newly recruited activated macrophages. Our studies demonstrate that C57BL/6 $CD8^+$ CTL lysed *Brucella*-infected macrophages; however, $CD8^+$ T cells from $A\beta^{-/-}$ mice failed to kill infected syngeneic macrophages in a similar fashion. In preliminary experiments, we detected reduced expression of B7-1 and B7-2 on Mac-1^+ spleen cells from $A\beta^{-/-}$ mice (data not shown). Therefore, one possible explanation for the minimal CTL activity observed in $A\beta^{-/-}$ $CD8^+$ T cells is the lack of MHC class II molecules that down-regulate the expression of costimulatory molecules like B7 on APC that are critical for cell-cell interaction and activation [40]. These results suggest that in normal mice, killing of infected targets by CTL may be another mechanism by which $CD8^+$ T cells control *B. abortus* infection. However, our results show that $A\beta^{-/-}$ $CD8^+$ T cells do not kill infected macrophages, but nevertheless, this mutant mouse strain controls the infection as rapidly as C57BL/6 mice. Therefore, we speculate that killing of infected target cells is not one of the most critical arms of the immune system to combat brucellosis.

In summary, our studies using MHC class I- and class II-deficient mice have helped to clarify the role of T cell subsets in murine brucellosis. We show unequivocal data demonstrating the predominant role played by $CD8^+$ T cells in controlling an intracellular bacterial infection. Moreover, these findings provide important insight into the regulatory and effector functions mediated by $CD8^+$ T cells to confer protective immunity.

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Immunization of mice with recombinant L7/L12 ribosomal protein confers protection against *Brucella abortus* infection

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BALB/c mice were immunized with the recombinant *Brucella abortus* L7/L12 ribosomal protein fused to maltose binding protein (MBP). Vaccinated animals mounted a specific immune response to the recombinant fusion protein as demonstrated by immunoblot analyses. Additionally, *B. abortus* L7/L12 ribosomal protein conferred a significant degree of protection when compared to mice vaccinated with adjuvant alone, adjuvant plus MBP or *B. abortus*. These results indicate that a recombinant *B. abortus* protein, previously identified as T-cell-reactive, engendered protective immunity to mice against brucellosis.

Key words:
[KWD]: *Brucella abortus*, recombinant vaccine, ribosomal protein

1. Introduction

Brucella abortus is a facultative intracellular bacterium that infects humans and domestic animals[1]. Brucellosis is an important zoonotic disease that causes abortion and infertility in cattle, and undulant fever, endocarditis, arthritis, and osteomyelitis in humans[2, 3, 4]. Infection in humans occurs through direct contact with infected animals or from ingestion of contaminated dairy products[5]. Thus, the control of brucellosis in animals is required for its control in humans. At present, live attenuated strain 19 is used to immunize cattle and the vaccine has three major disadvantages. First, strain 19 can cause abortion when administered to pregnant cattle[6]; second, strain 19 is pathogenic for humans[1]; and, lastly the vaccine induces antibodies in vaccinated cattle that interfere with the diagnosis of field infection[7]. Therefore, the development of more effective and safer vaccines is necessary for disease control.

Immunity to *B. abortus* is regarded as dependent on antigen (Ag)-specific-T-cell activation. $CD4^+$ T helper (Th) 1 subset and $CD8^+$ T cells are known to be involved in disease resolution[8, 9]. In addition, an important cell mediated mechanism responsible for protection is the gamma interferon (IFN-γ) up-regulation of macrophage anti-*Brucella* activity, the main host cellular reservoir for the bacterium[10]. Thus, the identification of specific *Brucella*-Ag that induce a type 1 T-cell subset response is a focused strategy to develop new genetically engineered vaccines.

The *B. abortus* L7/L12 ribosomal protein has been identified as an immunodominant Ag from this pathogen[11]. Also, we have characterized immunologically the nature of the T cell response to the L7/L12 ribosomal protein in its recombinant (r) form. Recombinant *B. abortus* L7/L12 ribosomal protein induced a Th 1 subset response from murine $CD4^+$ T cells featuring significant levels of IFN-γ production[12]. Ag that preferentially induce an IFN-γ producing Th1 subset response is a desirable subunit of any vaccine preparation against brucellosis. Furthermore, we have demonstrated that peripheral blood mononuclear (PBM) cells from *B. abortus* primed cattle are able to respond to the rL7/L12 *in vitro*, demonstrating that T-cell recognition of this particular Ag is not species specific[13]. Recently, others have identified the L7/L12 ribosomal protein from *B. melitensis* as a major component in the antigenicity of Brucellin INRA (Brucellergen) for Delayed-type hypersensitivity (DTH) in *Brucella*-sensitized guinea pigs[14]. Additionally, Skeiky *et al.* have isolated from *Leishmania braziliensis* a gene homologous to the eukaryotic ribosomal eIF4A gene and they have characterized its recombinant protein as a potent Ag capable of stimulating strong Th1-type responses as well as IL-12 production in

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leishmaniasis patients PBM cells[15].

Having characterized the recombinant *B. abortus* L7/L12 as a T-cell reactive ribosomal protein and knowing the immunological importance of ribosomal preparations[16], we decided to test the ability of such Ag in conferring protection to mice against brucellosis.

2. Material and methods

2.1. *Mice*

Female BALB/c mice were purchased at 8 weeks of age from Harlan Sprague–Dawley (Indianapolis, IN) and were used for immunization. All animals were housed in a Biosafety Level (BL)-3 facility and handled according to University of Wisconsin–Madison Research Animal Resource Center guidelines.

2.2. *Bacteria*

Brucella abortus, the live attenuated vaccine strain 19, was obtained from Barbara Martin (National Animal Disease Center, Ames, IA) at 2×10^{10} c.f.u. ml^{-1}.

2.3. *Immunization*

Five groups of 15 mice were immunized intraperitoneally (i.p.) with 0.2 ml of the following treatments:

(1) phosphate buffered saline (PBS);
(2) Immuneplus adjuvant system (Atlanta Biologicals, Norcross, GA);
(3) adjuvant containing 75 μg of maltose binding protein (MBP);
(4) adjuvant containing 100 μg of MBP-L7/L12 fusion protein; or
(5) *B. abortus* strain 19.

Recombinant L7/L12 comprises 25% of the fusion protein molecular mass, therefore, the animals vaccinated with MBP-L7/L12 received 25 mg of the specific *B. abortus* L7/L12 ribosomal protein[17, 18, 19]. To ensure that both mouse groups received equal quantity of MBP, we used different protein concentrations per dose containing the same MBP amounts to vaccinate each group. Briefly, animals were vaccinated with recombinant proteins in Immuneplus™ II (complete) on day 0 and in Immuneplus™-I (incomplete) on days 10 and 21. The negative control groups were similarly injected with PBS or adjuvant alone. The positive control group was vaccinated on day 0 with 5×10^4 c.f.u. *B. abortus* in 0.2 ml of PBS[20]. This immunization experiment was performed twice to confirm validity of the data.

2.4. *Gel electrophoresis and immunoblot analysis*

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed as described previously[13]. Briefly, non-cleaved and cleaved MBP-L7/L12 fusion protein were solubilized in sample buffer containing 2% SDS and 5% 2-mercaptoethanol and processed according to the method of Laemmli[21]. Before electrophoresis an equal volume of 2× concentrate sample buffer was added, and the mixture was boiled for 3 min and loaded onto the gel. Protein samples and molecular weight markers were analyzed on SDS–15% PAGE gel and visualized by Coomassie Blue staining. For western blot (immunoblot) analyses, the gel was electroblotted onto nitrocellulose at 75 V for 2 h. Transfer was carried out in 25 mM Tris–192 mM glycine–20% methanol. The blotted nitrocellulose was blocke with skim milk for 2 h. Then, serum from naive mice and mice immunized with the recombinant MBP-L7/L12 were used at a 1:500 dilution during incubation for 3 h (at room temperature). After reaction with the primary antibody, the blot was washed three times with TSBT [0.5 M NaCl–0.02 M Tris (pH 7.5), 0.05% Tween 20] and incubated for 1 h with goat anti-mouse immunoglobulin G-alkaline phosphatase conjugate (Promega Corp., Madison, WI) at a 1:10 000 dilution in TBST. Then the blot was washed three times with TBST, and the reaction was developed using nitroblue tetrazolium and BCIP (5-bromo-4-chloro-3-indolyl-1-phosphate) purchased from Promega.

2.5. *Challenge*

Mice were challenged with an i.v. injection of 1×10^6 c.f.u. *B. abortus* in 100 μl of sterile phosphate buffer saline (PBS), one week after the last immunization (on day 28).

2.6. *Quantitation of bacteria*

Seven, 14 and 30 days after challenged, five mice from each group were killed by cervical dislocation. Spleens were homogenized in plastic bags in sterile PBS using a Stomacher Lab Blender (Tekmar, Co., Cincinnati, OH), tenfold serial diluted, and plated on Brucella agar (Difco, Detroit, MI). Colonies were counted after incubation for 3 days at 37°C under 5% CO_2.

2.7. *Statistical analyses*

A mean value for each spleen count was obtained following log conversion. Statistical analyses were performed with Student's *t*-test using a computer software package MINITAB (Minitab Inc., State College, PA). Log units of protection were obtained by subtracting the mean c.f.u. for the proteins or *B. abortus* immunized mouse groups from the mean c.f.u. for the corresponding control groups that received adjuvant or PBS alone, respectively.

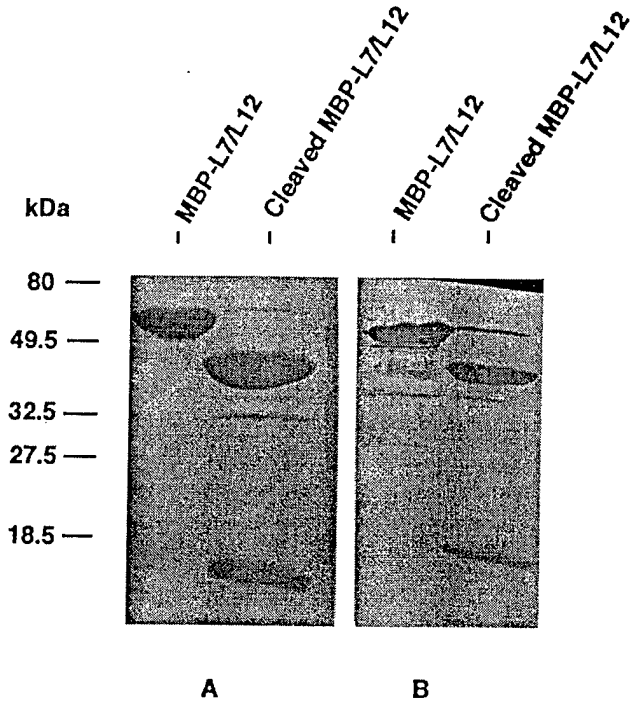

Fig. 1. SDS–PAGE profile and corresponding western blot analyses. (A) Coomassie Blue-stained SDS–15% PAGE of MBP-L7/L12 fusion protein and cleaved MBP-L7/L12. (B) Electrotransferred samples from SDS–PAGE were analyzed by immunoblotting with mouse anti-MBP-L7/L12 antibody

3. Results

3.1. *Analyses of antibody response to immunization with MBP-L7/L12*

To determine whether vaccinated animals mounted an immune response to the recombinant L7/L12 protein, SDS–PAGE and immunoblot analyses were performed. Fig. 1(B) illustrates a specific antibody response from animals immunized with recombinant MBP-L7/L12 fusion protein. Serum samples from MBP-L7/L12-immunized mice contained anti-MBP and anti-L7/L12 antibodies. As negative control, serum samples from naive mice were also tested and no anti-MBP-L7/L12 antibodies were detected (data not shown).

3.2. *Recombinant L7/L12 ribosomal protein engendered protection*

Three immunizations with 100 μg of the recombinant L7/L12 ribosomal protein fused to MBP protected mice against *B. abortus* infection at all three intervals analyzed ($P< 0.01$), while vaccination with 75 μg of MBP alone resulted in no protection ($P\leq 0.54$), Table 1. This result clearly demonstrates that an irrelevant *Escherichia coli* protein (MBP) does not protect mice against brucellosis. However, the rL7/L12 protein engendered substantial protective immunity (0.84–1.21 log units) to BALB/c mice against challenge when compared to the protection levels conferred to mice vaccinated with *B. abortus* S19 (1.1–2.85 log units). According to the kinetic study performed here, the greatest levels of protection observed in mice immunized with the rL7/L12 or *B. abortus* were at 2 and 4 weeks post-challenge when bacterial c.f.u. started to decline in mouse spleens.

4. Discussion

Alternative vaccines to strain 19 have been sought for many years with limited success[22, 23]. In previous studies, subunit vaccines have been used, but they were not highly effective in protecting animals from subsequent infection and disease[22, 24]. Alternatively, recombinant *B. abortus* proteins when used as immunogens have induced an increased humoral response but did not protect *Brucella*-challenged mice[25]. These previous results demonstrate that increased antibody response alone is not able to reduce *B. abortus* c.f.u. in mouse spleens. Therefore, *Brucella*-proteins that elicit a protective cellular response need to be investigated. As a first step towards the development of new genetically engineered vaccines, identifying the protective *B. abortus* proteins is of critical importance.

In this study, we have demonstrated that recombinant *B. abortus* L7/L12 ribosomal protein conferred protective immunity to BALB/c mice against brucellosis. The *B. abortus* L7/L12 ribosomal protein has been previously shown to be immunodominant in mice and cattle[11, 12].

Table 1. Level of protection conferred by immunization with the recombinant *B. abortus* L7/L12 ribosomal protein compared to adjuvant alone, MBP or *B. abortus*

Treatment group (n=5)	Log_{10} units of *B. abortus* in spleen (mean±S.E.)[a]			Log units of protection[b]		
	Time post-challenge					
	1 week	2 week	4 week	1 week	2 week	4 week
PBS	4.80±0.25	6.10±0.33	4.35±0.25	—	—	—
Adjuvant	4.84±0.33	6.08±0.38	4.15±0.31	—	—	—
Adjuvant+MBP	4.78±0.24	6.00±0.42	4.01±0.24	0.08*	0.10*	0.14*
Adjuvant+MBP-L7/L12	4.00±0.15	4.95±0.45	2.94±0.14	0.84**	1.13**	1.21**
B. abortus	3.70±0.12	3.25±0.25	1.70±0.12	1.10**	2.85**	2.65**

[a]The data are representative of two experiments performed separately with similar results. [b]* $P \geq 0.54$ (not significant); ** $P < 0.01$ (significant) compared to control groups

Knowing that protection to brucellosis is dependent on cell-mediated immunity[8, 9], our present finding is consistent with previous observations that L7/L12 elicits T-cell activation *in vitro*[12, 13].

Although, humoral immunity is not reportedly as protective as cellular immunity[25], a specific antibody response to MBP-L7/L12 fusion protein was observed in vaccinated BALB/c mice. The anti-L7/L12 antibody detection by immunoblot analyses, was the approach used to confirm successful vaccination. Mice immunized with recombinant MBP-L7/L12 conferred 0.84–1.21 log units of protection when compared to 1.21–2.85 log units from *B. abortus* vaccinated group. Therefore, this protein is a logical candidate to be included in a future subunit vaccine against brucellosis. Besides protection, another immunological feature of this protein is its ability to elicit DTH[14]. Additionally, *B. abortus* L7/L12 ribosomal protein may be used as a diagnostic tool in an improved skin test which can be optimized for the diagnosis of brucellosis.

Even though, the L7/L12 ribosomal protein afforded protective immunity, we postulate that a multiple subunit vaccine would provide superior protection to that engendered by vaccination with single *B. abortus* protein. As observed in malaria and leprosy models, solid protective immunity requires immunization with several parasite proteins rather than a single moiety[26, 27]. Therefore, an immunogen comprised of a single molecule may not be sufficient to confer full protection. To our knowledge, this study is the first report of protective immunity engendered by immunization with a single recombinant *B. abortus* T-cell-reactive ribosomal protein. Also, our data with *B. abortus* confirms the work of others who reported that ribosomal preparations from other pathogens are highly protective vaccines[16]. Furthermore, additional *B. abortus* proteins that induce cell-mediated protection need to be identified to compose a multiple protein subunit vaccine.

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Cellular Immunology

RECOMBINANT *BRUCELLA ABORTUS* PROTEINS THAT INDUCE PROLIFERATION AND GAMMA-INTERFERON SECRETION BY $CD4^+$ T CELLS FROM *BRUCELLA*-VACCINATED MICE, AND DELAYED-TYPE HYPERSENSITIVITY IN SENSITIZED GUINEA PIGS

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Running title: Cellular responses to recombinant *B. abortus* antigens

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ABSTRACT

Optimal protective immunity to *Brucella abortus* infection is dependent on a coordinate interaction between different T cell subsets which leads to an antigen (Ag)-specific T lymphocyte mediated activation of macrophages, the main cellular reservoir for the bacterium. As an initial step in the identification of bacterial proteins that mediate cellular immunity, we have subcloned the *B. abortus ssb*, *uvrA*, *GroES*, and *GroEL* genes into the prokaryotic expression vector pMAL-c2 using PCR. *Escherichia coli* DH5α was transformed with the pMAL-ssb, pMAL-uvrA, pMAL-GroES, and pMAL-GroEL constructs separately, and gene expression was induced by IPTG (isopropyl-β-D-thiogalactopyranoside). The resulting fusion proteins were purified by affinity chromatography and confirmed by Western blot analysis using an anti-maltose-binding protein antibody. Furthermore, we have examined the pattern of T helper (Th) cell response from vaccinated BALB/c mice after *in vitro* stimulation with the recombinant (r) fusion proteins. In addition to T cell proliferative responses, $CD4^+$ T cells were tested for interleukin-2 (IL-2), IL-4, and gamma interferon (IFN-γ) secretion. Primed $CD4^+$ T cells proliferated to the rUvrA, rGroES, and rGroEL, but not to rSsb. The cytokine profile of the

proliferating cells was characteristic of a Th1 type, as we detected IL-2 and IFN-γ but not IL-4 in the T cell culture supernatants. The recombinant *B. abortus* proteins were also screened *in vivo* to their ability to elicit DTH reaction in *Brucella*-sensitized guinea pigs. Moreover, the results of this study suggest that *B. abortus* rUvrA, rGroES, and rGroEL might be important sources of potentially protective molecules.

INTRODUCTION

Resistance to facultative intracellular bacterial pathogens, such as *B. abortus*, depends on acquired cell-mediated immunity (CMI) and activation of macrophages by T lymphocytes (1). Additionally, an important cell mediated mechanism responsible for protection is the gamma interferon (IFN-γ) up-regulation of macrophage anti-*Brucella* activity, the main host cellular reservoir for the bacterium (2). $CD4^+$ T helper 1 subset and $CD8^+$ T cells are known to be involved in disease resolution (3-5). Th1 $CD4^+$ T cells are responsible for macrophage activation and attraction of inflammatory effector cells and therefore have been suggested to play a role in acquired cellular resistance (6). The ability of specific antigen (Ag) to induce preferentially a Th1 or Th2 subset response is an important aspect for the development of molecular vaccines against pathogens (7). Identification and characterization of dominant T-cell Ag from *B. abortus* is therefore an important strategy in the search for protective subunit vaccines and specific diagnostic reagents.

Few *B. abortus* genes have been cloned and the proteins that they encode have not been characterized as T-cell Ag (8-12). In addition, the potential for these proteins to elicit a protective cellular response against

B. abortus infection has not been investigated. Therefore, the identity of *B. abortus* Ag which induce the formation of protective T cells is yet unknown.

Heat shock proteins (hsp) are known as chaperones or stress proteins with important biological functions in protein biogenesis. The production of hsp is greatly enhanced by stress stimuli such as rise in temperature, exposure to toxic oxygen radicals, and nutritional deficiencies. Hsp GroEL for example, is involved in the folding, assembly, and transport of factors required for growth under these conditions (13). Additionally, hsp were identified as immunodominant Ag in a variety of microbial pathogens (14-20). *B. abortus GroES* and *GroEL* genes have been cloned (21-23). Interestingly, cattle vaccinated with attenuated *B. abortus* strain 19 (22), and cattle and mice infected with virulent strain 2308 (23) mount an antibody response to the recombinant GroEL. However, the role of these *B. abortus* proteins as T-cell reactive Ag have not been investigated.

Previously, we have isolated *B. abortus ssb* and *uvrA* genes using primed bovine T lymphocytes as probes (24). Knowing that *B. abortus* primed T cells of immune mice respond strongly to Ag in the cytoplasmic protein fraction prepared from this bacteria (25), we decided to investigate

further the role of the rSsb and rUvrA proteins as specific stimulators of T lymphocyte activation.

This study describes initially, the subcloning of *B. abortus ssb*, *uvrA*, *GroES*, and *GroEL* genes and their expression in *Escherichia coli*. Additionally, the nature of the T lymphocyte response to the recombinant proteins was characterized *in vitro* by $CD4^+$ T cell proliferative response and by the pattern of cytokines produced, as well as *in vivo* based on DTH activity in *Brucella*-sensitized guinea pigs.

MATERIAL AND METHODS

Mice

Female BALB/c mice were purchased at 8 weeks of age from Harlan Sprague-Dawley (Indianapolis, IN) and were used for experimental infection. All animals were housed in a Biosafety Level (BL)-3 facility and handled according to University of Wisconsin-Madison Research Animal Resource Center Guidelines.

Bacteria

Brucella abortus, the live attenuated vaccine strain 19, was obtained from the National Animal Disease Center (Ames, IA) at $2x10^{10}$ colony-forming units (CFU)/ml. For use as Ag in the T-cell cultures, the bacteria were killed by γ-irradiation with ^{137} Cs (250000 rads).

Vaccination of mice

BALB/c mice received an intraperitoneal (i.p.) injection of $1x10^5$ CFU *B. abortus* in 200 µl of sterile phosphate-buffered saline (PBS). Six weeks post-infection, the animals were killed by cervical dislocation and the spleen harvested for $CD4^+$ T cell preparation.

Subcloning of *Brucella* genes in pMAL-c2

The *B. abortus ssb* and *uvrA* as well as the *GroES* and *GroEL* heat-shock genes (kindly provided by J. Mayfield, Iowa State University, Ames, IA) were amplified by PCR and subcloned into the expression vector pMAL-c2 (New England Bio Labs, Beverly, MA). Primers containing one artificial restriction site at each end, were designed according to the nucleotide sequence of each gene (21, 24) using the OLIGO Primer Analysis Software computer program (National Biosciences, Inc., Plymouth, MN). The primer sequences are shown in the Table 1. PCR reaction was performed with a 50 µl volume containing 100 ng of DNA template from each gene, 1 µM of each primer, 2.5 mM $MgCl_2$, 200 µM (each) deoxynucleoside triphosphates, 1x PCR buffer and 2.5 U of *Amplitaq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT). PCR amplification was conducted with a DNA thermal cycler (Perkin-Elmer Cetus) using the conditions described elsewhere (26). PCR amplified product was purified by QIAEX II gel extraction kit (Qiagen Inc., Chatsworth, CA) and digested with the appropriate restriction endonucleases. After digestion, the PCR product was purified again by the same procedure and ligated to the predigested pMAL-c2 vector, using DNA ligase (Promega Corp., Madison,

WI). The ligation reaction was used to transform *E. coli* DH5α, and single recombinant clones were selected. Plasmid DNA was extracted by Magic Miniprep (Promega) and digested with the appropriate enzymes to verify the presence of the insert.

Expression of *Brucella*-genes and recombinant protein production

E. coli clones harboring separately the pMAL-ssb, pMAL-uvrA, pMAL-GroES, and pMAL-GroEL constructs were selected and gene expression was induced by 0.6 mM IPTG (isopropyl-β-thiogalactopyranoside). The bacterial cells were lysed by freezing/thawing plus lysozyme treatment and centrifuged at 9000 g for 30 min. The supernatant was diluted 1:5 in PBS and loaded on an amylose column to affinity purify the fusion proteins. The purified fusion proteins were concentrated in PBS using centriprep concentrators (Amicon Inc., Beverly, MA), and the protein concentration determined by the Bio-Rad protein assay (Bio-Rad, Hercules, CA) according to manufacturer's directions (26).

SDS-PAGE and Western-blot

For SDS-PAGE, *E. coli* lysates carrying the fusion proteins and MBP only, were solubilized in sample buffer containing 2% SDS and 5% 2-mercaptoethanol (ME) and processed as described previously (26). Before

electrophoresis, an equal volume of 2x concentrated sample buffer was added, the mixture boiled for 3 min, and loaded onto the gel. Proteins were analyzed on a 12% SDS-PAGE and visualized by Coomassie staining.

For Western blot analysis, the gels were electroblotted onto nitrocellulose at 75 V for 2 h. Transfers were carried out in 25 mM Tris-192 mM glycine-20% methanol. The blotted nitrocellulose was blocked with skim milk for 2 h. Then, rabbit anti-MBP serum was used at a 1:5,000 dilution during incubation for 2 h at room temperature. After reaction with the primary antibody, the blots were washed three times with TBST (0.5 M NaCl-0.02 M Tris [pH 7.5], 0.05% Tween 20) and incubated for 1 h with a goat anti-rabbit immunoglobulin G-alkaline phosphatase conjugate (Promega) at a 1:10,000 dilution in TBST. Then, the blots were washed three times with TBST, and the reactions were developed by using nitroblue tetrazolium and BCIP (5-bromo-4-chloro-3-indolyl-1-phosphate) purchased from Promega (27).

Purification of $CD4^+$ T cells

Single cell suspensions were prepared from the spleen of naive and vaccinated mice. Splenocytes were isolated by density gradient centrifugation using Fico-Lite^{TM} (Atlanta Biologicals, Norcross, GA). Cells were washed three times with sterile PBS containing 100 U/ml of

penicillin, 100 µg/ml of streptomycin and 50 µg/ml of gentamicin and resuspended in ice-cold RPMI 1640 (Sigma Chemical Co., St. Louis, MO) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml of streptomycin, 25 mM HEPES, $5x10^{-2}$ M 2-ME and 10% heat-inactivated fetal bovine serum (Sigma). $CD4^+$ T cells were purified using an affinity chromatography column (Biotex Laboratories Inc., Alberta, Canada) by a process of negative selection with rat anti-mouse Ly2 (CD8a) mAb and polyclonal goat anti-mouse IgG, according to manufacturer's directions. Flow cytometry analysis confirmed that >91% of the negatively selected splenocytes expressed CD4 molecules on their surface.

T-cell proliferation assay

Murine $CD4^+$ T cells ($1x10^5$) from naive and vaccinated animals were stimulated with 25 µg/ml of the recombinant fusion proteins (the average optimal concentration was determined after testing 1-100 µg/ml), with $1x10^7$ γ-irradiated CFU *B. abortus* or concanavalin A (2.5 µg/ml) as a T-cell-activating control, in a final volume of 200 µl/well in a 96-well plate. Syngeneic irradiated (2000 rads) spleen cells ($4x10^5$) from naive animals were added as antigen presenting cells (APC) to the $CD4^+$ T cells. The cells

were cultured for 72 h and pulsed with 1 μCi [^{3}H] thymidine (Amersham Corp., Arlington Heights, IL) in each well for 8 h. The cells were harvested onto glass fiber filters, and the radioactivity was determined in a liquid scintillation counter. Assays were performed in triplicate.

Cytokine analysis

Following the same protocol used to stimulate T lymphocytes in the T-cell proliferation assay, culture supernatants of $CD4^+$ T cells from vaccinated animals were tested for the presence of cytokines using ELISA to mouse IL-2, IL-4, and IFN-γ (Endogen, Boston, MA). The assays were performed in duplicate according to manufacturer's directions.

Skin reaction test

Two male Hartley guinea pigs were sensitized by subcutaneous injection of 10^6 *B. melitensis* Rev1 organisms per animal. Three weeks later, 1 μg of each recombinant fusion protein MBP-UvrA, MBP-GroES, MBP-GroEL, or 1 μg of MBP alone (negative control) or Brucellergen (positive control) were injected intradermally in a final volume of 100 μl. Diameters (in millimeters) of the areas of erythema were read 24 h later. The immediate nonspecific response was checked after 4 h as described

elsewhere (28). Two naive Hartley guinea pigs were also injected with the same protein preparations to check for nonspecific reactions.

RESULTS

Expression of *B. abortus ssb*, *uvrA*, *GroES*, and *GroEL* genes in *E. coli*

To obtain large amounts of the recombinant proteins, *B. abortus ssb*, *uvrA*, *GroES*, and *GroEL* genes were expressed in *E. coli* using the pMAL-c2 expression vector system. This vector expresses foreign sequences as a fusion protein with the 42.7 kDa MBP, as previously described (26). *E. coli* lysates containing each separated fusion protein were purified by affinity chromatography. The resulting products were analyzed on a 12% SDS-PAGE (Fig. 1A). The molecular mass for each fusion protein observed on SDS-PAGE (MBP-Ssb [61.2 kDa], MBP-UvrA [67.7 kDa], MBP-GroES [52.7 kDa], and MBP-GroEL [102.7 kDa]) correlates to the expected size of each *Brucella* protein separated from MBP (Ssb-18.5 kDa, UvrA-25 kDa, GroES-10 kDa, and GroEL-60 kDa). To confirm that the purified products were the recombinant *B. abortus* fusion proteins, Western blot analysis was performed using rabbit anti-MBP antibody. Figure 1B shows the anti-MBP antibody recognition of the MBP-Ssb, MBP-UvrA, MBP-GroES, and MBP-GroEL fusion proteins.

$CD4^+$ T-cell proliferative response

$CD4^+$ T cell culture were isolated and purified from spleens of mice vaccinated with live *B. abortus* strain 19, and the Ag-specific T-cell response to the different recombinant *Brucella* fusion proteins were compared to the response of cells from naive animals. $CD4^+$ T cells from primed animals responded to whole γ-irradiated *B. abortus* as well as the fusion proteins MBP-UvrA, MBP-GroES, and MBP-GroEL, but not to MBP-Ssb when these preparations were used as Ag (Fig. 2). However, when $CD4^+$ T cells from naive mice were used no significant T-cell proliferative response was detected. Taken together, these results indicate that Th cells from vaccinated mice respond specifically to stimulation with the recombinant *Brucella* UvrA, GroES, and GroEL or whole *B. abortus*. As a negative control, MBP alone was used as Ag in the same concentration as the other proteins with no proliferation detected (data not shown). The lack of MBP reactivity was confirmed when MBP-Ssb was used and no Th cell proliferative response was detected (Fig. 2).

Cytokine production to recombinant *Brucella* antigens

T-lymphocyte activation to these *Brucella* proteins was also assayed by the presence of IL-2, IL-4, and IFN-γ on Ag-activated $CD4^+$ T cell culture supernatants. A $CD4^+$ T-cell cytokine profile to rMBP-UvrA, rMBP-GroES, and rMBP-GroEL was demonstrated by the presence of secreted IL-2 and IFN-γ but not IL-4 (Fig. 3 A, B, and C). This cytokine pattern induced by the recombinant proteins is similar to the profile detected when γ-irradiated *B. abortus* was used as Ag (29). The MBP-Ssb fusion protein did not induce lymphocyte proliferation and cytokine production by $CD4^+$ T cells. On the other hand, recombinant *Brucella* GroEL heat-shock protein induced the highest levels of IL-2 and IFN-γ secretion compared to the other Ag used.

Our recent study using quantitative RT-PCR has demonstrated that *B. abortus* strain 19 induces a type 1 cytokine profile *in vivo* in mouse splenocytes (5). Therefore, the cytokine pattern shown in Fig. 3 after *in vitro* Ag stimulation, probably reflects the activation of Ag-specific Th1 clones previously differentiated after vaccination with live *B. abortus* strain 19. Knowing the importance of IFN-γ to enhance resistance to intracellular pathogens such as *B. abortus* (30, 31), the identification of Ag

from this pathogen that induce a Th1 subset response is pivotal to new strategies on vaccine development. However, the development of a subunit vaccine may well require delivery of T-cell Ag in the context of adjuvants or delivery systems that bias the local immune response toward IFN-γ production.

Taken together, these results demonstrate that a dominant Th1 type cytokine profile is detected from $CD4^+$ T cells from *Brucella*-vaccinated mice following stimulation with recombinant UvrA, GroES, and GroEL Ag.

DTH activity of the recombinant proteins

B. abortus and *B. melitensis* share 98% of homology at DNA level (32). Therefore, we used *B. melitensis* sensitized guinea pigs as a previously optimized system to measure DTH activity (28). The antigenicity of the MBP-UvrA, MBP-GroES, and MBP-GroEL was compared to Brucellergen (positive control) and MBP (negative control) in a pilot study. No detectable DTH reaction to the MBP-GroES or MBP alone was observed in sensitized guinea pigs, Table 2. However, when recombinant MBP-UvrA or MBP-GroEL were injected, a positive DTH reaction was observed compared to the controls. The *Brucella* GroEL protein induced a very strong DTH reaction (10 to 12 mm) compared to the Brucellergen (20 mm). Two naive guinea pigs that were inoculated with the fusion proteins, MBP, or

Brucellergen did not show any reaction (data not shown). DTH activity as another mechanism to measure cell-mediated immunity *in vivo*, also confirms the recombinant *B. abortus* GroEL heat-shock protein as the strongest stimulus to primed T lymphocytes among the Ag studied.

DISCUSSION

In this study, we have subcloned and expressed the *B. abortus ssb*, *uvrA*, *GroES* and *GroEL* genes. Recombinant *B. abortus* fusion proteins produced were screened for the ability to induce $CD4^+$ T cell proliferation, cytokine production, and DTH activity. These are three different mechanisms to measure *in vitro* and *in vivo* T lymphocyte activation.

Immunity to intracellular bacteria such as *B. abortus* is considered to be mediated by cell-mediated immunity (CMI) (33). As products of CMI, cytokines are key molecules that play major roles in shaping the development of protective or noncurative immune responses (34). Among them, IFN-γ is an important cytokine that up-regulates macrophage anti-*Brucella* activity and is considered crucial for protection against *B. abortus* infection (2, 31). Additionally, IFN-γ is an important mediator in effecting the delayed-type inflammatory response given by Th1 clones (35). DTH is another expression of cellular immunity. In a mouse model, only Th1 clones were capable of producing an Ag-specific and MHC-restricted inflammation reaction with the characteristics of DTH (36). Furthermore, inflammatory reactions including DTH response in brucellosis and listeriosis have been shown to be mediated exclusively by $CD4^+$ T cells (4).

The recombinant *B. abortus* UvrA, GroES, and GroEL proteins induced $CD4^+$ T cell proliferation, and IL-2 and IFN-γ secretion following *in vitro* stimulation. However, only rUvrA and rGroEL elicited a DTH response in *B. melitensis* sensitized guinea pigs. Among the Ag studied, the rGroEL heat shock protein induced the highest level of T-cell proliferation, IL-2 and IFN-γ production, and it was the strongest DTH stimulus for sensitized guinea pigs. These results, heightened our interest in determining the importance of this Ag in protective immunity.

Evidence is accumulating that hsp are major antigens of many pathogens (37). Because of abundance or peharps other reasons, hsp become prominent antigens that trigger a major portion of the immune system repertoire. Stress-induced GroEL protein synthesis is observed following phagocytosis of *Brucellae* by macrophages (13). In mice immunized with *Mycobacterium tuberculosis*, T cells are strongly activated against hsp 65 (37). Remarkably, a high degree of protection against challenge with *M. bovis* BCG or *M. tuberculosis* H37Rv in mice vaccinated with the *M. leprae* hsp 65, suggests the potential usefulness of this Ag in a vaccine against pathogenic mycobacteria (38). In the case of leishmaniasis, PBMC from infected patients proliferate vigorously, and produce IL-2 and

IFN-γ to recombinant *Leishmania braziliensis* hsp 83 (39). Additionally, the *Mycobacterium leprae* GroES heat shock protein has been reported as a major T cell antigen to PBMC cells from infected patients (40). These reports confirm hsp as important Ag involved in T lymphocyte activation, and as efficient inducers of potential protective immunity.

Production of reactive oxygen intermediates is the mechanism used by macrophages to kill intracellular *B. abortus* (41). DNA repair enzymes, such as UvrA, are important in repairing DNA lesions resulting from oxidative damage (42). Therefore, we hypothesize that in a hostile environment inside a phagocytic cell, *B. abortus* enhances expression of UvrA and possibly other DNA repair enzymes as a protection mechanism. The stress response in bacteria is characterized by adjustments in the activity and synthesis of proteins associated with many different processes. Because bacterial protein synthesis may be augmented during intracellular infection or due to preferential Ag presentation of peptides which are critical to bacterial survival, primed $CD4^+$ T cells are activated following *in vitro* stimulation with rUvrA.

Previously, we have shown that a *B. abortus* ribosomal rL7/L12 protein induced a Th1 subset response from primed murine $CD4^+$ T cells

(29), and its homolog from *B. melitensis* has been reported to be the major component in the antigenicity of Brucellergen for DTH reaction (28). Recently, we demonstrated that this Ag has induced protective immunity to BALB/c mice against *B. abortus* infection (43). Similarly, others have shown that *Yersinia* rGroEL-reactive $\alpha\beta$ TCR^+ $CD4^+$ Th1 cells mediated protection to mice against infection by human pathogenic *Y. enterocolitica* serotypes 08 and 03 (44). In leishmaniasis, a *Leishmania chagasi* recombinant Ag that stimulated murine splenic T cells to secrete the Th1-type cytokine IFN-γ, engendered partial protection to BALB/c mice against challenge with the live organism (45). These results lead us to speculate that there is a correlation between induction of Th1 type of response, DTH activity, and protection. Our next experiment will be to test the ability of the *B. abortus* rUvrA, rGroES, and rGroEL to confer protection to mice against experimental brucellosis.

In summary, we have demonstrated that recombinant *B. abortus* proteins UvrA, GroES, and GroEL are efficient inducers of Th1 lymphocyte activity, as analyzed by the production of IL-2 and IFN-γ. As such, they are particularly important sources of potentially protective vaccine candidates. Additionally, this study suggests that selection of antigenic

determinants from proteins that are highly conserved among species of pathogens is a desirable approach in the search for protective epitopes. Future studies will define antigenic determinants of *B. abortus* proteins and evaluate them in a mouse model for protection against experimental brucellosis.

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Table 1. Primer sequences used for DNA amplification and subcloning of the *GroES*, and *GroEL* genes.

Gene	Primer sequence 5' → 3'	Orientation
ssb	GGGGGATCCATGGCTGGTAGCGTCAAC	sense
	GCGGGGCTGCAGTCAGAACGGAATTTCATC	anti-sense
uvrA	GGGGGATCCATGAGCGATCAGAAATTC	sense
	GGGTCTAGAACAGGTTTCGAGACTGTC	anti-sense
GroES	ACCAAGGAATTCACCATGGCTGATATCAAG	sense
	CGCGGGTCTAGATTTTTAGCCGACAATACC	anti-sense
GroEL	TCCCAGGAATTCAAAATGGCTGCAAAAGAC	sense
	AGCGGGTCTAGATTCTTAGAAGTCCATGCC	anti-sense

Table 2. DTH analysis of *B. abortus* rUvrA, rGroES, and rGroEL proteins in *B* pigs

Antigens	Animals	
	#1	#2
	D1 x D2 [a]	
MBP	Neg [b]	Neg
MBP-UvrA	7.5 x 7.5	0.5 x 0.5
MBP-GroES	Neg	Neg
MBP-GroEL	11.5 x 11.5	10 x 10
Brucellergen	20 x 29	20 x 20

[a] D1 x D2 represents the eliptical diameters of erythema (mm)

[b] Neg, Negative

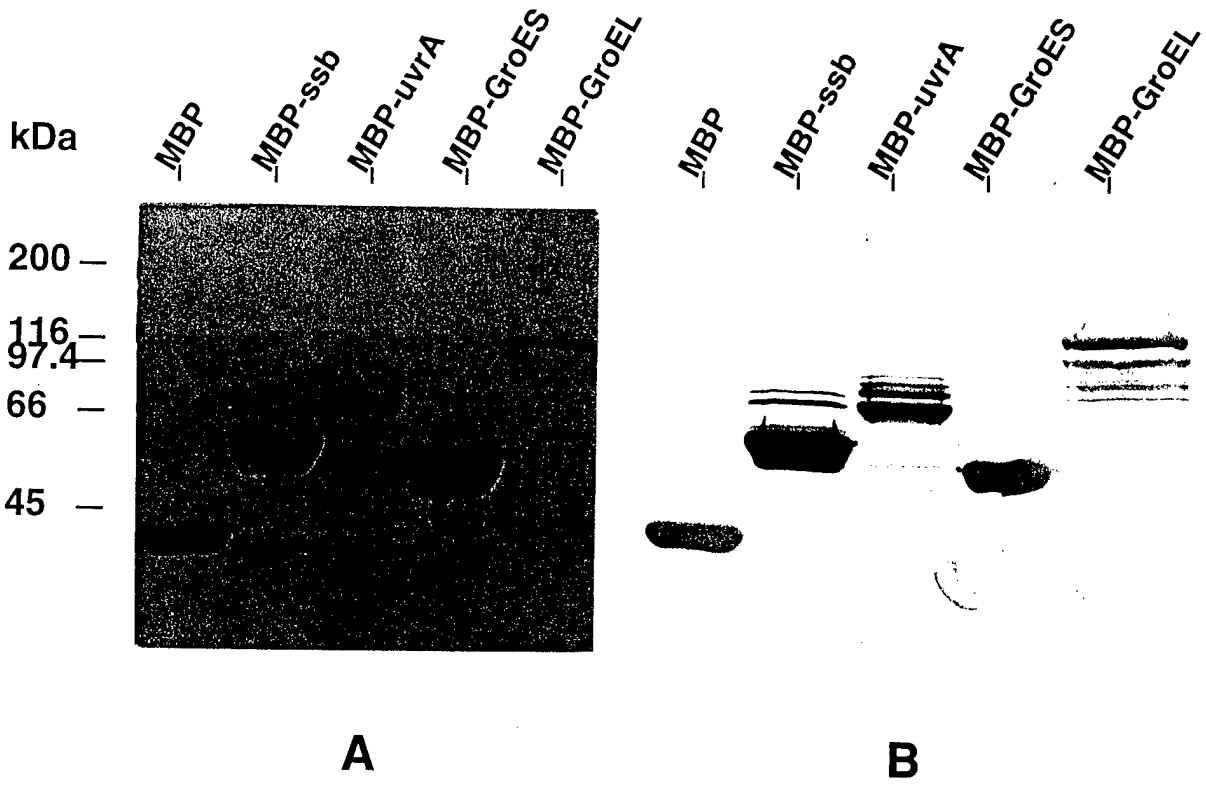


FIG 1. (A) Coomassie blue-stained SDS-12% PAGE profile of MBP, MBP-Ssb, MBP-UvrA, MBP-GroES, and MBP-GroEL recombinant fusion proteins. (B) Electrotransferred samples from SDS-PAGE were analyzed by immunoblotting using rabbit anti-MBP antibody.

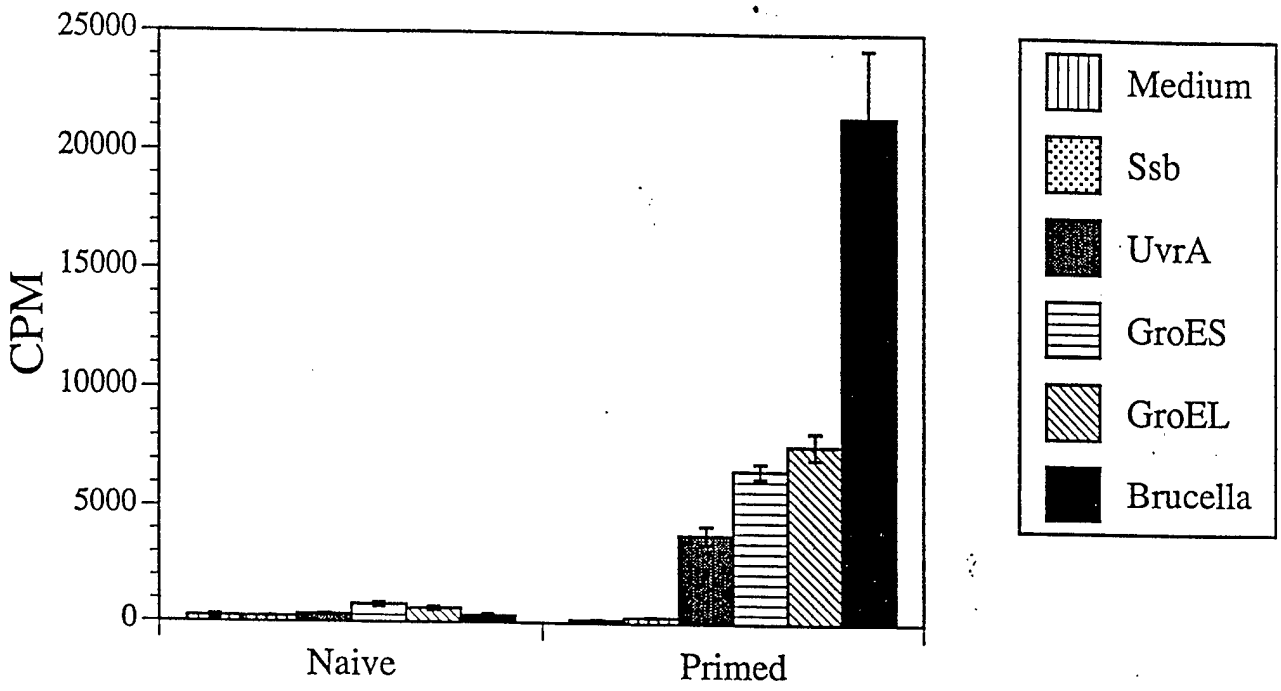


FIG 2. Proliferative responses of $CD4^+$ T-cell cultures derived from *B. abortus*-primed and naive BALB/c mice. Th cells were *in vitro* stimulated with rSsb, rUvrA, rGroES, rGroEL, γ-irradiated *B. abortus*, or Con A. Con A-stimulated cells from primed and naive animals had cpm above 100,000 (data not shown). Results are expressed as means cpm. Error bars indicate SE of the means.

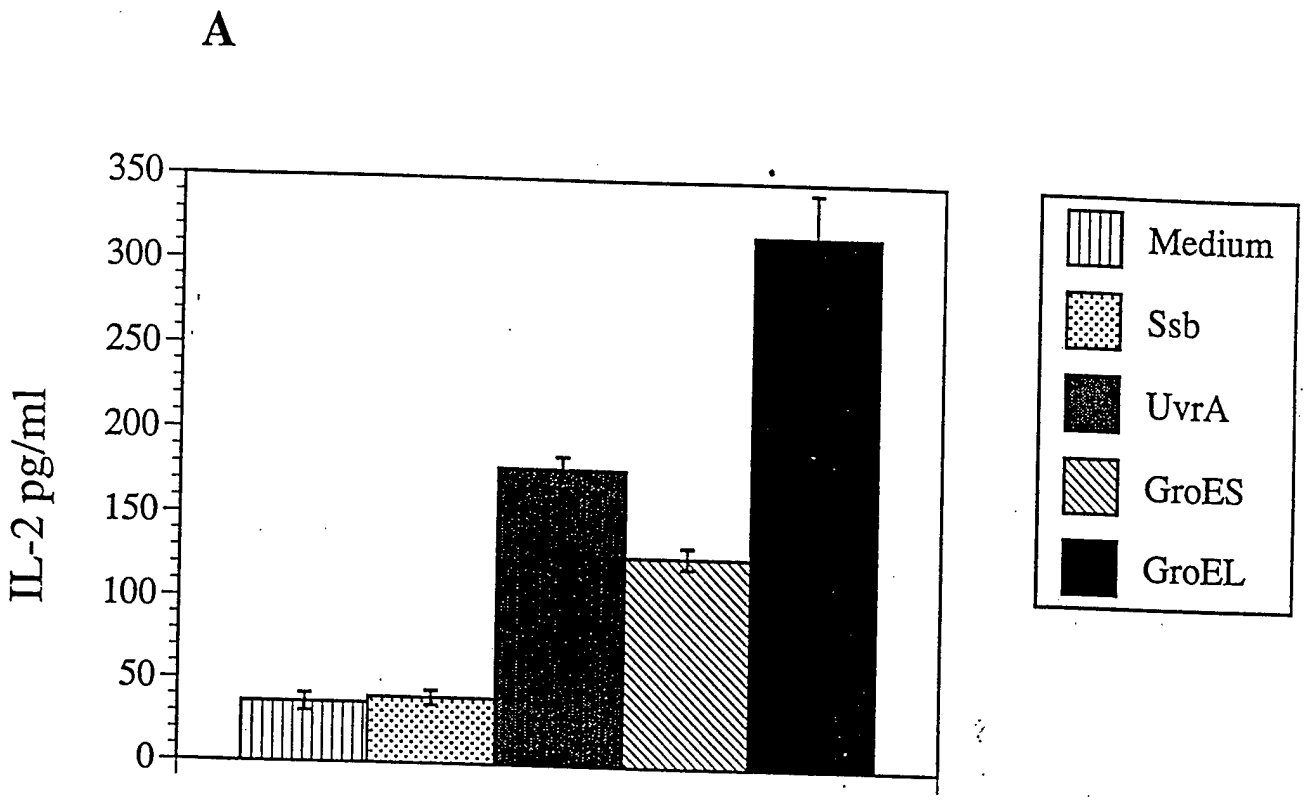
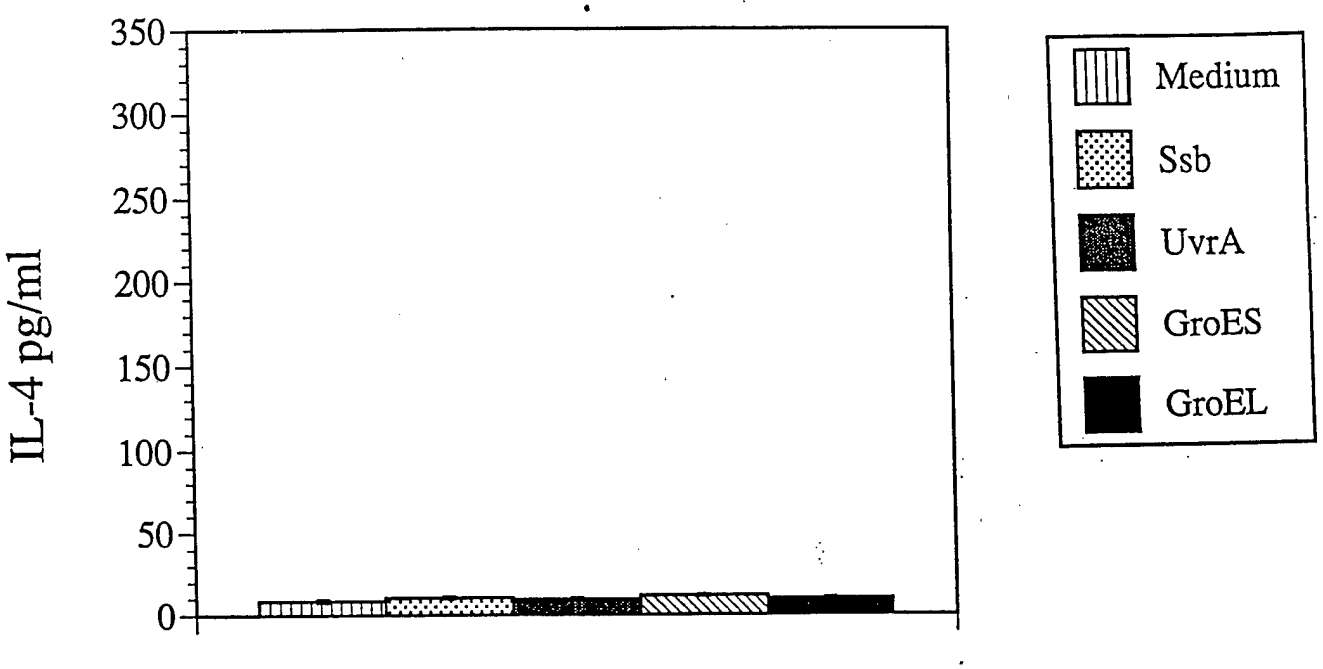


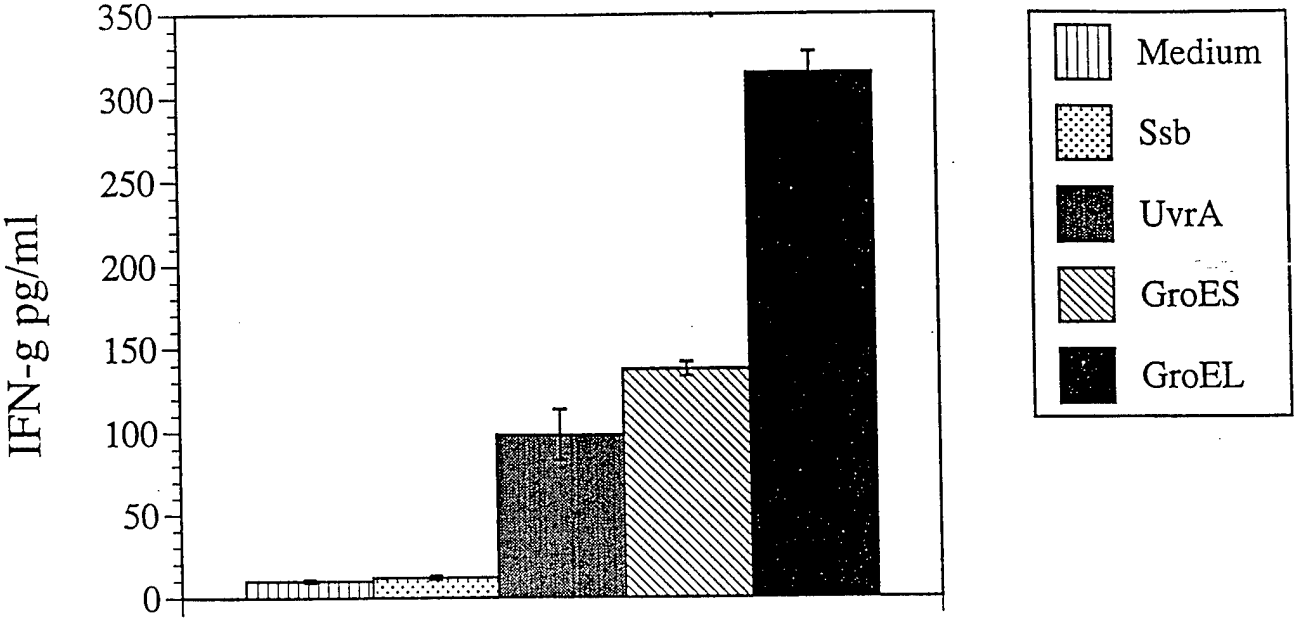
FIG 3. Cytokine responses of $CD4^+$ T cells from *B. abortus*-primed BALB/c mice restimulated *in vitro* with rSsb, rUvrA, rGroES, rGroEL, or Con A for 48 h. Levels of IL-2 (A), IL-4 (B), and IFN-γ (C) present in Th cell culture supernatants were assayed by ELISA. Supernatant of Th cells cultured with Con A as a positive control had the following cytokine amounts: IL-2 (6295±24 pg/ml), IL-4 (416±22 pg/ml), and IFN-γ (38240±890 pg/ml) (data not shown). Data are expressed as means ± SE.

FIG. 3 Cont.

B


350
300
250
200
150
100
50
0
IL-4 pg/ml
Medium
Ssb
UvrA
GroES
GroEL

C


350
300
250
200
150
100
50
0
IFN-g pg/ml
Medium
Ssb
UvrA
GroES
GroEL